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13953

Tests for the RH Factor with Guinea Pig Immune Sera.*

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In a previous paper,[1] a method of preparing immune sera in guinea pigs for the human blood property Rh was given. The technic has been found to be improved by the following modification.

Full-grown guinea pigs are injected intraperitoneally 5 times at intervals of about 5 days, with each dose consisting of the washed cells from 2 cc of blood from rhesus monkeys. A week after the last injection the animals are bled. Satisfactory sera may be pooled and preserved with merthiolate 1:5000.

For the tests, the sera (inactivated, if fresh) were diluted 10 times with saline and absorbed for one hour at room temperature with one-tenth volume of blood sediment containing equal parts of A_1 and B blood. Whether the absorbing blood is Rh-positive or Rh-negative seems to make little or no difference, showing that the antibodies responsible for the distinctive reaction are not readily absorbed by Rh-positive blood and have less affinity for human cells than the other antibodies present in the serum. The tests are carried out and read by inspection of the sediment, as previously described. The tests could also conveniently be carried out with dilutions (*e.g.* 1:4) of the absorbed fluid, and in fact in this way the difference in appearance between positive and negative sediments may even be more conspicuous.

The advantage of this modification, entailing longer continued immunization, appears to be the higher proportion of usable sera obtained. Probably owing to the greater content of species (as well as Rh) antibodies, the procedure of simple dilution was found to be inadequate, and absorption was necessary in order to obtain a sharp distinction between positive and negative bloods.

* One of the authors (A. S. W.) was aided by a grant from the Carnegie Corporation, through the Committee on Human Heredity of the National Research Council.

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13954

Volume of Parathyroid Glands in Relation to Dietary Calcium and Phosphorus.

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Enlargement of the parathyroid glands has been observed in the spontaneous rickets of rats[1] and humans[2,3] and has been experimentally produced in a variety of species by diets low in calcium.[4–9] However, typical rickets is produced experimentally by diets low, not in calcium, but in phosphorus. Not low phosphorus diets, but on the contrary, both *high* phosphate diets[10] and parenteral phosphate injections[11,12] have been found to cause parathyroid enlargement. Recently Ham and co-workers[9] essayed to determine, by dietary experiments, what alteration in blood calcium or phosphate constituted the essential physiological stimulus to parathyroid enlargement. These authors confirmed the fact that a low calcium diet (Steenbock) with its attendant hypocalcemia caused enlargement of the glands in young rats. They found no enlargement on a Steenbock low phosphorus diet with hypophosphatemia and marked rickets. Finally by adding a mixture of calcium phosphate and phosphoric acid to a "normal" diet they succeeded in raising the average serum phosphate of 4 rats 2 mg % above that of 3 controls without changing the serum calcium and without increasing parathyroid size. They concluded that "hypocalcemia, instead of hyperphosphatemia, is the primary cause of physiological hypertrophy of the parathyroid glands."

We have attempted to confirm and extend these rather meager data. Albino rats of the Sherman strain have been placed on variations of the Steenbock diet* at weaning (age 3 weeks). After 4 weeks they were killed by bleeding from the heart under ether anesthesia. Pooled specimens of serum were analyzed† for calcium (Clark-Collip) and inorganic phosphate (Fiske-Subbarow). The parathyroids were fixed in Bouin's fluid, embedded in paraffin, serially sectioned at 10 micra and their volumes determined by tracings and planimeter measurements.[6] Bones were fixed and decalcified in Mueller's fluid and studied histologically. A comparison has been made between the glands of rats on the low phosphorus diet and of

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* Basal low Ca diet: Ground yellow corn 76 parts, gluten flour 20, sodium chloride 1. This diet contained 355 mg Ca per kg by analysis of Dr. Edgar Miller. One drop Lederle's Vitamin B complex was given 6 times weekly to each rat. For low P diet, 3 parts $CaCO_3$ were added. The rats on Vitamin D supplement received 1 drop Squibb's Viosterol in oil (170 I.R.U.) twice weekly.

† We are indebted to Mrs. Ethel B. Gutmann for some of the chemical analyses.

TABLE I.
Effect of Dietary Calcium and Phosphate upon Parathyroid Volume.

Diet	No. rats	Mean body wt (g)	Mean parathyroid vol. (mm^3)	Mean ratio parathyroid/ body (mm^3/100 g)	Serum Ca (mg%)	Serum Inorganic phosphate (mg%)
Stock (aged 30 days)	14	50	.100	.200		
Stock ('' 50 '')	10	160	.120	.075		
Stock ('' 50 '' — restricted intake)	9	63	.091	.144	10.8	6.7
Steenbock Low P	16	50.8	.060	.116	11.8	2.0
Steenbock Low P + 400 mg% K_2HPO_4	10	60	.054	.095	11.9	4.4
Steenbock Low P + 800 mg% K_2HPO_4	9	51.2	.087	.174	10.3	7.1
Steenbock Low P + 1500 mg% K_2HPO_4	16	47.4	.145	.310	10.8	10.2
Steenbock Low P + 3000 mg% K_2HPO_4	8	53	.223	.425	7.3	6.1
Steenbock Low P + Vitamin D	10	49.2	.047	.098	14.1	5.5
Steenbock Low Ca + 800 mg% K_2HPO_4	10	41	.336	.724	5.9	7.8
Steenbock Low Ca + Vitamin D	9	61	.136	.236	10.7	8.3

those on a stock diet.‡

Results. Growth was poor on all the modifications of the Steenbock diet. Severe rickets occurred in all the animals on the low P diet. This was completely prevented by the vitamin D supplement. Rickets was mild and inconstant in the animals receiving 400 and 800 mg supplements of K_2HPO_4 and absent in those receiving 1500 mg. Growth was poorest in the animals on the low calcium diet and these animals all had severe low calcium type of rickets.

The chart and table show that the parathyroids were tremendously enlarged in the low calcium group. The glands were very small in the low P group and the addition of vitamin D made them even smaller while raising both the serum calcium and phosphate. The addition of graduated amounts of K_2HPO_4 to the low P diet caused a corresponding increase in parathyroid size and serum phosphate without causing a uniform change in serum calcium. The glands were smaller in rats on the low P diet than in control rats on the stock diet either (1) of the same age, (2) of the same body weight but younger, or (3) of the same age and weight due to restricted feeding.

Discussion. The difficulty of comparing organ sizes in animals of different age or weight is obvious. In animals of the same age, the *ratio* of organ to body weight is frequently used to advantage where the body weights are widely different. The inadequacy of the Steenbock diet for the production of normal growth even with vitamin B supplements and the marked effect of alterations in dietary calcium and phosphorus on growth have influenced our results. For these reasons the effect of growth retardation through restricted feeding of the stock diet was determined. It is apparent from the table that parathyroid volume and body weight are not proportionately reduced by this expedient. The unpredictable degree of influence of general inanition in the rats on the experimental diet therefore rendered this ratio of limited usefulness in comparing the results.

In spite of this handicap, certain facts are clearly demonstrated by the results. These are: (1) the *increase* in parathyroid volume

‡ Rockland rat diet: Yellow corn, hulled barley, hulled oats, whole wheat, soy bean meal, meat scraps, powdered whole milk, alfalfa meal, NaCl, $CaCO_3$. This contains about 2.2% Ca and 1.3% P.

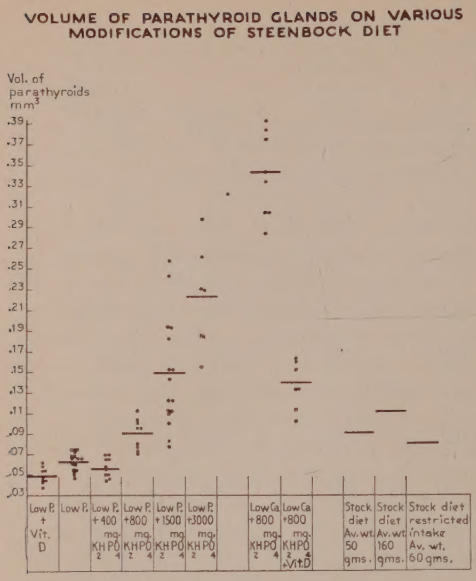

CHART 1.

on a low calcium diet, (2) the *decrease* in parathyroid volume resulting from a low phosphorus diet, and (3) the rough proportionality between parathyroid volume and phosphorus content of the diet when the calcium content is unaltered.

The conclusion of Ham *et al.*[9] that "hyperphosphatemia in the absence of hypocalcemia does not cause physiological hypertrophy of the parathyroid gland" does not apply to our results. We have observed increases in parathyroid volume with increasing serum phosphate concentrations in the absence of hypocalcemia as determined at the end of the experiment. It is possible, perhaps likely, that the maintenance of the normal Ca level in the blood under these conditions is an expression of a compensatory overactivity of the parathyroids.

Conclusions. (1) A low calcium diet with its attendant hypocalcemia causes marked enlargement of the rat's parathyroid (confirming past reports). (2) A low phosphorus, high calcium diet with consequent hypophosphatemia causes a significant reduction in parathyroid volume in comparison with stock diet controls. (3) Addition of graduated increments of K_2HPO_4 to the low phosphorus, high calcium diet increases the serum phosphate proportionately and causes a corresponding increase in parathyroid volume. (4) Large doses of viosterol further reduce the size of the parathyroid on the low phosphorus diet, at the same time raising both serum calcium and phosphate. Viosterol also inhibits the hyperplasia produced by a low calcium diet. (5) No simple formula has yet been devised to relate parathyroid volume to the serum calcium and phosphate concentration under all dietary conditions.

We wish to express our thanks to Mrs. Claudia Schogoleff for technical assistance.

13955 P

Action of *o*-Iodosobenzoic Acid on Certain Bacteria.

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Although *o*-iodosobenzoic acid was studied with regard to its bactericidal action by Loevenhart and Grove[1] there seems to have been little if any work done on this subject since then. These authors presented, along with their results pertaining to physiological effects, evidence that *o*-iodosobenzoic acid and related compounds had definite *in vitro* bactericidal action upon organisms of the colon group including *B. typhosus* and upon *B. pyocyaneus* and *Staphylococcus aureus*. Bacteriological studies *in vivo* were not performed. Sodium *o*-iodosobenzoate was found to be highly toxic to the animal organism when administered subcutaneously and intraperitoneally.

Recent work has indicated that *o*-iodosobenzoate at pH 7 may be used for the quantitative estimation of —SH groups whether in compounds such as cysteine and glutathione[2] or in proteins such as denatured hen's egg albumin and urease[3,4] according to the process:

$$2\ RSH + \overline{O}OC—C_6H_4\ OI \rightarrow \begin{matrix} R—S \\ R—S \end{matrix} + IC_6H_4COO^- + H_2O$$

While the following experiments can in no sense be considered as final, the evidence at hand suggests there may be a correlation between the reproduction and survival of certain bacteria and the presence of —SH groups (*cf.*[5,6]) although it is recognized that iodosobenzoate is not entirely specific for the sulfhydryl group. Irrespective of theory it may prove worthwhile to study further the possible use of locally applied *o*-iodosobenzoic acid in the treatment of infected wounds. The work is presented in its present stage since the author will not be able to do further experimental work for the duration.

Experiments with E. coli. With Fildes synthetic ammonium lactate medium and concentrations of organisms of about 1000/cc it was found that *o*-iodosobenzoate in a concentration of 0.5 mg % completely prevented growth for as long as 72 hr, and as growth was not obtained on replating it was concluded that the effect was bactericidal rather than bacteriostatic. Sulfanilamide controls showed definite growth after replating. A concentration of 1.0 mg % completely inhibited the growth of as many as 5×10^7 organisms per cc while 0.25 mg % permitted only very slight growth of 5×10^3 organisms per cc after 48 hr.

Experiments with beta hemolytic streptococci. Beef broth infusion medium was used in the following, *o*-iodosobenzoate being added from a sterile solution. Two strains of organisms were employed; the first the C-203 strain and the second an organism recently isolated from a case of scarlet fever. With 4000 organisms per cc a concentration of 10 mg % of *o*-iodosobenzoate had a slightly more marked effect than controls with the same concentration of sulfadiazine. Again here the effect of the *o*-iodosobenzoate was bactericidal rather than bacteriostatic.

Animal experiments (mice). Intraperitoneal injections of 120 mg of *o*-iodosobenzoic acid dissolved in an equivalent amount of NaOH in a total volume of about 1.5 cc resulted in death within 15 min with symptoms of shock; on examination there was found about 5 cc of gelatinous clear exudate with scattered petechial hemorrhages on the

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mesenteries and intestines. With amounts of 15 mg or less in about 1.0 cc the animals, while at first becoming sick, eventually recovered; no residua were noted on autopsy several days later. Because of the acute toxicity of the drug when administered in this fashion a series was run in which the free acid was placed either subcutaneously through an incision or injected as an emulsion in about 1.0 cc of water in the loose tissues about the neck. Amounts below 30 mg per 30 g mouse were fairly well tolerated; the main reaction again was an accumulation of sterile gelatinous exudate which was later resorbed.

A known virulent C-203 strain was injected subcutaneously along side about 15 mg of *o*-iodosobenzoic acid and at no time was there evidence of infection in the 6 animals used; the controls without *o*-iodosobenzoic acid all died within 48 hr. In the presence of the same amount of drug as many as 5×10^7 organisms had no more effect than 1×10^3 and all cultures from the site of the injections and the heart's blood failed to show hemolytic streptococci. In a series of animals receiving 60 mg of the drug all were dead within 48 hr as a result of the toxic effects of the drug but none showed any signs of infection on autopsy or culture.

Conclusions. 1. *o*-iodosobenzoate *in vitro* apparently has as marked if not more marked an effect than the sulfonamides on *E. coli* and certain hemolytic streptococci. 2. The effect is bactericidal rather than bacteriostatic. 3. It is conceivable that the drug may act by interruption of some catalytic process dependent on intact —SH groups. 4. Locally *o*-iodosobenzoic acid apparently did control in these experiments what should have been an overwhelming infection. The *o*-iodosobenzoate which is much more soluble seems to be too toxic to be used locally.

These data suggest that *o*-iodosobenzoic acid may be worth further study as a possible bactericidal agent in the treatment of infected wounds.

It is a pleasure to thank Dr. Charles L. Fox, Jr., for many valuable suggestions in the bacteriological technics involved.

13956

Injurious Action of Pitressin on the Rat Testis.*

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Some of the biological effects of adrenocorticotropic pituitary preparations suggested the presence of a pressor substance. A temporary prostration of the rats was observed invariably to follow injections of high levels of these partially purified preparations. Coincidentally it was noted that there were injurious effects of high levels of these preparations on the testes of normal and hypophysectomized males. Two methods have been employed in the effort to determine whether the injurious effects on the testes may be referred to pressor principles. First, attempts were made to remove any pressor substance from ACT preparations and to compare the biological properties of the crude and purer materials. This phase of the subject will be discussed later. Second, effects of the pressor principle itself on the testis have been studied and are here reported.

As a maximum of 5 dog pressor units per mg have been found in ACT preparations, pitressin† was injected into normal 40-day-

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† Kindly supplied by Parke, Davis and Co.

TABLE I.
Effects of 5 Pressor Units (Dog) Daily of Pitressin on Reproductive System of Normal Immature Male Rats.

Treatment	Age at onset, days	No. of rats	Injection period, days*	Organ wt		
				Testes, mg	Seminal vesicles, mg	Prostate, mg
Pitressin	26	5	23	1248	48	189
Control at onset, 25 da	—	5	—	284	11	52
Control at autopsy, 49 da	—	7	—	1734	99	211
Pitressin	40	5	15	1458	161	271
Control at onset, 41-44 da	—	58	—	1206	41	132
Control at autopsy, 55-56 da	—	36	—	2305	231	282

*Injections were intraperitoneal and were given daily except Sunday, hence 13 injections were given in the 15-day period, 18 injections in the 23-day period.

old male rats for a period of 15 days, at a dose of 5 pressor units daily. As can be seen in Table I, the testis was definitely decreased in weight by this treatment—almost halved.‡ Spermatozoa were absent in the epididymis, or were present in small numbers only, whereas the epididymis of the normal controls (55 days old) contained abundant spermatozoa. Histologically, the testes showed varying degrees of injury. The normal testis of the rat at this age contains tubules of uniform caliber, which have not yet attained their maximum diameter, and mature spermatozoa or spermatids are found in practically all tubules. The interstitial cells have acquired a considerable body of cytoplasm and are epithelioid rather than "connective tissue like." After pitressin treatment, the tubules were usually lined by spermatocytes; sometimes spermatids were present and occasionally a few abnormal spermatozoa. A variable number of tubules, in some cases most tubules, showed not only retardation of development but marked evidences of injury. Some showed sloughing of the entire epithelial investiture down to the basement membrane, and in a few instances complete disintegration of tubules occurred. The interstitial tissue, though normal in appearance, did not seem to be functioning at a normal rate, judged by the weight of the accessories. Development of the seminal vesicles was definitely retarded, that of the prostate only slightly.

‡ Body weight increase was also retarded by about 20 g. (Body weights were the same at onset, the normals gained 80 g, the injected 64 g.)

As can be seen in Table I, similar results were obtained in 26-day-old normal males injected daily for 23 days with 5 pressor units.

The injurious effects of pitressin on the testis were also demonstrable in the hypophysectomized rat. Testis weights were smaller in hypophysectomized rats receiving either 1.0 or 0.1 units of pitressin daily. Histological examination made clear that this decrease was due to injurious effects on the tubules. The testis of the rat hypophysectomized at 40 days of age when allowed to regress without treatment for 15 days decreases in weight far below that characteristic at the time of operation. Tubules become uniformly smaller; spermatids disappear; the number of layers of spermatocytes is reduced, although some healthy, adherent spermatocytes of the first order are still present in most tubules after this postoperative interval. By this time also, characteristic changes in the chromatin of the Leydig cells have occurred. The testes of hypophysectomized rats which had received pitressin from the time of operation did not show this orderly picture. Superimposed upon the regular regressive changes were obvious signs of injury, such as death and extensive desquamation of tubular epithelium. Interstitial cells were not further affected, nor was the usual regression of seminal vesicles and prostate influenced. (Table II).

The behavior of animals receiving pitressin

TABLE II.
Effect of Pitressin Injected from Time of Operation on Regressing Reproductive System of Male Rats Hypophysectomized at 40 Days of Age.

Treatment	No. of rats	Daily dose* (IP)	Injection period, days	Organ wt		
				Testes, mg	Seminal vesicles, mg	Prostate, mg
Pitressin	4	1.0	15	360	16	47
"	4	0.1	15	322	14	42
Hypophysectomized controls, 55 da old	32	—	—	431	17	51

*Dog pressor units.

was observed to see if the prostration effect of ACT preparations was given by the doses of pitressin which injured the testes. The 5 pressor unit dose, corresponding to the amount of contamination in the usual dose of ACT, 1 mg, had a corresponding effect on the animal. The drowsy appearance, labored breathing and slowed righting reflex lasted for 10-20 min after each injection; 45 min after injection the animal appeared entirely normal.

Conclusion. Pitressin in high dosage has been found to be injurious to the tubular epithelium of the testis of the rat. In interpretation of the biological effects of extracts of pituitary substance it is therefore extremely important to keep in mind the possibility of this contaminant.

13957

The Attenuating Effect of Promin on Virulence of the Tubercle Bacillus.

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It has been shown previously that p,p'-diaminodiphenylsulfone-N-N'-dextrose sulfonate (promin) in concentrations of 20 mg % inhibits the growth of the tubercle bacillus in culture media.[1] Also promin administered orally[1] or in the diet[2] retards the tuberculous process in guinea pigs. To determine the mechanism of the action of promin upon the virulence of the tubercle bacillus experiments were designed to ascertain the effect of prolonged cultivation of the bacillus on the medium containing the drug in low concentrations. Changes in virulence of the strain were determined by the degree of invasiveness of the attenuated strain, (a) when inoculated upon the chorio-allantoic membrane of the chick embryo and (b) when inoculated intraperitoneally into guinea pigs.

Methods. The A 27 strain of human tubercle bacilli was selected since this strain was known to have a moderately high degree of virulence. The bacilli were grown in a culture medium consisting of beef bouillon plus 5% glycerine with 10 mg % of promin. Preliminary experiments had shown that the strain could not survive this concentration of promin continuously; hence, after the second transfer into the medium containing promin, the strain was returned to the control medium. After the strain had recovered its capacity to grow in the control medium, it was again placed in the medium containing promin. Finally, the suspension for inoculation was made from bacilli grown in the

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2 Feldman, W. H., Hinshaw, H. C., and Moses, H. E., *Am. Rev. Tuberculosis*, 1942, **45**, 303.

promin-free culture medium. The total attenuation period with promin in the divers experiments varied from 24 hr to 104 days, comprising 1 to 4 transfers in promin and 2 to 3 in control medium. The ultimate decrease in the virulence of the strain may be considered, therefore, a result of the accumulative effect of the drug after prolonged treatment.

In all experiments bacillary suspensions were prepared by weighing the bacilli and diluting to the desired concentration with normal saline so that .2 cc contained 1 mg of bacilli. Finely dispersed suspensions were obtained by rotating the weighed bacilli in a White Ball Bacteria Grinder for 30 min. Each egg received 1 mg of bacilli on the outer surface of the chorio-allantoic membrane. The technic of inoculation of the membranes, their fixation, the embedding and staining, and evaluation of the extent and degree of tuberculous involvement was the same as previously described.[3] The average extent of tubercle formation in all membranes of each experiment was compared with a similar group inoculated with bacilli of the same strain cultured in control medium. The differences indicate the extent of attenuation achieved.

As an additional check on the relative virulence of the control strain and the promin-treated strain of tubercle bacilli, 2 groups consisting of 15 guinea pigs each and weighing on the average 350 g were inoculated intraperitoneally with 0.5 mg tubercle bacilli, the one with a suspension prepared from the strain of bacilli which had been treated with promin for 104 days and the other with a suspension of untreated bacilli. The animals were weighed once a week. This experiment was terminated after 50 days when all survivors were killed and autopsied. The extent of tuberculous involvement in the omentum and glands, spleen, liver, lungs, peritoneum and kidneys was noted and the "tuberculosis index" evaluated as previously described.[3]

Results. A. Chick Embryo Technic. Table I summarizes the results of the experiments with the chorio-allantoic membrane. With the exception of experiments 59 and 69 the promin-treated bacilli were returned to a normal medium before preparing the suspension for inoculation. In experiments 59 and

[3] Emmart, E. W., and Smith, M. I., *Am. Rev. Tuberculosis*, in press.

TABLE I.

Effect of Attenuation of Virulence of Tubercle Bacilli of the A 27 Strain After Treatment with Promin.

Exp. No.	No. of chick embryos inoculated	No. of embryos surviving	Total days of strain of bacilli in promin media	Total days of strain in control media	% macroscopic tubercles	Microscopic observations: Avg degree of cellular proliferation and aggregation, evaluated on basis of 0 to 4			
						Epithelium	Eosinophils	Monocytes	Tubercle formation
I. Chorio-Allantoic Membranes Inoculated with Promin-Treated Bacilli.									
59	27	13	1	0	57	1.0	1.0	1.4	0.4
69	30	8	1	0	41	0.6	1.6	1.5	0.6
52	25	8	22	90	75	1.0	0.8	1.1	0.8
49	27	9	54	31	66	2.0	1.3	1.7	1.3
50	24	5	54	52	50	1.0	1.5	1.2	0.7
53	24	8	54	65	100	2.3	2.0	3.0	2.6
54	22	5	54	64	100	2.0	2.2	3.0	2.8
74	24	21	104	103	80	1.3	1.3	1.7	1.3
Avg						1.4	1.4	1.8	1.3
II. Chorio-Allantoic Membrane Inoculated with Bacilli from Control Media.									
22	33	8	0	Continuously	100	2.2	2.6	3.6	3.8
51	24	18	0	"	95	1.6	1.7	2.2	1.9
55	18	4	0	"	100	1.0	2.0	2.2	2.0
70	36	18	0	"	100	1.5	2.1	2.5	2.1
75	24	19	0	"	100	1.8	2.4	3.0	3.0
Avg						1.6	2.1	2.7	2.5

TABLE II.

A Comparison of Degree of Tubercle Development in Membranes Inoculated with Promin-Treated Tubercle Bacilli and with Bacilli Grown on the Control Medium.
— None, + Slight, ++ Moderate, +++ Advanced, ++++ Extensive.

Embryo No.	Macroscopic tubercles	Degree of microscopic cellular proliferation and aggregation: Epithelium	Eosinophils	Monocytes	Tubercle development
I. Chorio-Allantoic Membranes Inoculated with Tubercle Bacilli Cultured for 104 Days on Media Containing Promin—Exp. 74.					
1	+	+++	+	+	+
2	+	+++	+++	++	++++
3	+	—	—	—	+
4	+	++	+++	+++	++
5	+	+	++	+++	+
6	+	+	++	+++	++
8	+++	++	+++	++++	+++
9	+	+	—	+	+
11	+	+	+	++	+
12	+	+	—	+	+
14	—	+	+	+	—
15	+	+	+	+	+
16	+	+	+	+	+
18	—	+	—	+	+
19	+++	++	+++	++++	+++
20	—	+	+	+	+
22	+	+	++	++	+
23	+	++	+	+	+
24	—	+	+	+	—
Avg	1.0	1.3	1.3	1.7	1.3
II. Chorio-Allantoic Membranes Inoculated with Tubercle Bacilli from Control Cultures—Exp. 75.					
2	++++	++	+++	++++	++++
3	++++	+	++	++++	++++
4	+++	+	++	+++	++
5	+	+	+	+	+
6	++++	+++	+++	++++	++++
9	++++	+++	+++	++++	++++
10	+++	++++	+++	+++	++++
14	++	+	++	+++	++
15	+++	++	+++	++++	++++
16	+	+	+	+	+
17	++++	++	+++	++++	++++
18	++++	++	++++	++++	++++
20	++	+	+	+	+
21	++++	++	++++	++++	++++
22	+	—	—	—	+
23	++++	+++	++++	++++	++++
Avg	3.0	1.8	2.4	3.0	3.0

69 the suspension was prepared from tubercle bacilli grown on a normal medium but subjected to 10 mg % promin for 24 hr at 37°C before inoculation. In these 2 experiments with bacilli treated with promin 24 hr, the surviving eggs had an average of 49% tubercle formation. Usually in 6 days membranes inoculated with 1 mg of normal control A 27 bacilli had 100% gross tubercles. The results from experiments 59 and 69 represent tuberculocidal action rather than attenuation which probably explains the low incidence of tubercle formation in these 2 experiments.

One attenuation experiment carried for 22 days in promin and 90 days in control resulted in 75% tubercle formation. Other cultures carried 54 days in promin and for increasing lengths of time in control medium varied from 50 to 100% in the production of tubercles. The tubercles, however, were usually small, fibrous and often not more than several aggregates of epithelioid cells.

Of the surviving embryos the membranes inoculated with promin-treated bacilli 74% were either negative or possessed tubercles in early stages of development, while only 26% showed large tubercles. In the control strain with an incidence of nearly 100% tubercle formation in almost every instance the tubercles were large, fully developed and showed caseation and necrosis. The difference in the virulence of the promin-treated strain and the control strain was well illustrated after 104 days of treatment with promin. This strain after culturing in control medium for 29 days was suspended in normal saline and implanted on the membranes of 24 embryos. Fifty-eight per cent of the membranes so inoculated showed a slight tubercle development, 12% extensive tubercle development and 8% none at all. In the control experiments all membranes had tubercles; of these, 75% showed extensive tubercle development and 25% slight tubercle development. The data of this experiment are given in detail in Table II.

Microscopic examination of the sections of the chorio-allantoic membranes revealed marked differences in the virulence of the treated and non-treated strain of bacilli. The degree of epithelial proliferation, the number and extent of the aggregations of eosinophils and monocytes and the degree of tubercle development was generally less pronounced in the membranes implanted with promin-treated bacilli.

The average incidence of tubercle formation in the 2 series of experiments was nearly twice as high in the controls as compared with the promin-treated strain (Table I) and nearly 3 times as high when a comparison is made on the basis of tubercle formation on individual membranes implanted with bacilli treated with promin for 104 days (Table II). Differences in the several types of cellular proliferation were not the same in both series of experiments. Generally the differences in the extent of proliferation of the surface epithelium were least marked while those of monocytic proliferation were the most pronounced.

B. Guinea Pig Experiments. As an additional check on the relative difference in the

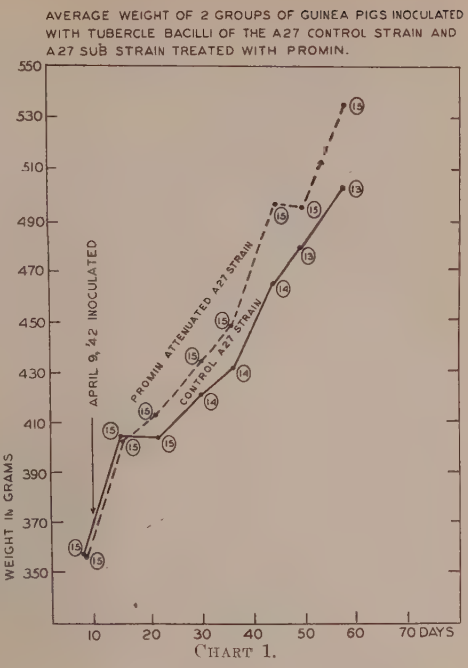

CHART 1.

virulence of the promin-treated strain of bacilli as compared with a control strain, a suspension prepared from a strain of bacilli treated 104 days with promin and one from a control strain were inoculated into 2 series of guinea pigs. Chart 1 shows graphically the differences in weights of these 2 series of guinea pigs. Two weeks after the inoculations the series of guinea pigs inoculated with the control strain lost weight more rapidly than the animals inoculated with the promin attenuated strain. This difference was maintained throughout the experiment. Fifty days after inoculation, when the experiment was terminated, the difference between the average weights of the guinea pigs inoculated with the promin-treated strain and the control strain was 37 g. The divergence of the 2 curves was accentuated as the animals in the control group developed symptoms of the more advanced stages of the disease.

In the series of animals inoculated with promin-treated bacilli none died within the experimental period. In the control group 1 died on the 23rd day and another on the

42nd day, and the rest were killed at the termination of the experiment. Upon autopsy, the degree of tuberculous involvement in the lungs, omentum, liver, spleen, and peritoneum and kidneys was evaluated on the basis of a possible maximum of 20 as previously described.[3] The control group showed a tuberculosis index of 9.0 as compared with an index of 6.6 for the group inoculated with promin-treated bacilli. From the differences in the weight curves as well as from the tuberculosis index of the autopsied animals it appears that the results of inoculation of the treated and untreated strains into guinea pigs parallel the results obtained when the same strains were implanted on the chorio-allantoic membrane of the chick embryo.

Conclusions. 1. Experiments are described to show that prolonged cultivation of tubercle bacilli on a promin-containing medium attenuates their virulence. 2. Under the experimental conditions the reduction in virulence in the promin-treated strain persisted after the strain was returned to control media. 3. The change in virulence can be demonstrated both by implantation on the chorio-allantoic membrane of the chick embryo as well as by inoculation into guinea pigs. 4. The attenuating action of promin is slight, this being probably related to the relatively low bacteriostatic action of this drug against the tubercle bacillus. It seems likely that more promising results might be obtained by the application of this method to more active bacteriostatic agents.

13958

Action of Alkaline Acriflavine Solution on *Bacterium salmonicida* and Trout Eggs.*

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Trout furunculosis is a costly, widespread, epizoötic bacteremia, that annually destroys millions of salmon and trout.[1] The disease occurs in trout hatcheries and rivers in at least 8 European countries, in Canada, and in more than one-fourth of the United States. Trout furunculosis is caused by *Bacterium salmonicida* Lehmann and Neumann, a gram-negative non-spore-forming rod which shows deeply-staining, bipolar bodies; the taxomic position of this bacterium has not been determined.

Among the agencies by which furunculosis may be disseminated are trout ova, which in practical fish culture are freely transported from contaminated hatcheries and spawning beds to clean hatcheries. In fish hatcheries, eggs are usually incubated in headwaters; and thus may be a source of infection to fish downstream. The interior of living trout eggs is believed to be bacteriologically sterile.[2,3] Only the dead eggs and the exterior of living ones can be considered as sources of infection. The former may readily be removed manually. If, therefore, the exterior of the living ova can be disinfected, eggs may be placed in headwaters in hatcheries, without danger to fish downstream.

* Subsidized by and carried out in the laboratories of the Wisconsin Conservation Department. Technical assistance contributed by the Fisheries Research Project, Work Projects Administration, Madison, Wisconsin.

1 Mackie, T. J., and Menzies, W. J. M., *J. Comp. Path. and Ther.*, 1938, **51**, 225.

2 Plehn, M., *Centl. Bakt.* (etc.), Abt. Orig., 1911, **60**, 609.

3 Williamson, I. J. F., *Fisheries, Scotland, Salmon Fish*, 1929, No. 1, His Majesty's Stationery Office, Edinburgh, 1929.

Blake[4] compared a number of disinfectants as to their toxicity for living trout ova and *Bact. salmonicida*. She recommended a 20-min dip in a solution of 500 parts per million (p.p.m.) neutral acriflavine (2,8-diamino 10 methyl acridinium chloride) as an effective disinfecting treatment which was harmless to trout eggs and lethal for *Bact. salmonicida*. Browning *et al.*,[5] Michaelis and Hayashi,[6] and Eggerth,[7] however, show that the bactericidal action of acriflavine is greatly reduced in neutral and acid solution. Gee and Sarles[8] recently compared the action of 19 disinfectants as to their toxicity for trout eggs and to *Bact. salmonicida*. They calculated a prophylactic index for each disinfectant, *i.e.* the ratio of germicidal power to the toxicity to eggs. They found that when used as herein described, acriflavine surpasses every product tested except sulfomerthiolate.

Experimental. Disinfection studies were made by means of the United States Food and Drugs Administration[9] method for testing disinfectants modified as follows: The medium was made by treating 500 g of ground, whole, fresh carp with 1 l of tap water for 2 hr at 70°C. The infusion was cooled, and the fat was swept off with gauze. The supernatant fluid was poured or pressed through several thicknesses of gauze and made up to 1 l. The infusion was autoclaved to precipitate the heat labile proteins and the fluid again filtered through gauze and restored to volume. One per cent of peptone (Parke Davis) was added and the pH adjusted electrometrically to 7.7. For semi-solid medium 0.4% of agar-agar powder and for solid medium 1.5% agar-agar was added. Agar media were used for subculturing the bacterium after treatment with acriflavine because of the marked adsorptive effect of agar on acriflavine.[7]

Stock cultures of *Bact. salmonicida* were carried on fish infusion agar slopes at 15°C and were transferred monthly. Work cultures were made up fresh each month from the month-old agar slope and were grown in tubes of 5 ml of fish infusion broth at 15°. These were transferred daily by pipetting 0.1 ml of 24-hr culture to a fresh tube. In resistance to acriflavine, this broth culture was shown to be equal to that grown in the standard methods broth made with special Armour's peptone. Except where otherwise designated, 24-hr broth cultures containing approximately 100,000,000 bacteria per milliliter were used in the disinfection tests. All tests were run at 10°.

Solutions of 500 p.p.m. neutral acriflavine U.S.P. were prepared with a naturally acid water (pH 6.2) with soft water in which carbon dioxide had been bubbled (pH 5.7), and soft water in which trout eggs had respired (pH 6.4). In all these solutions *Bact. salmonicida* survived longer than 30 min. Next, using Clark[10] buffer, 500 p.p.m. acriflavine solutions were prepared at pH 5.0, 5.5, 6.0, etc. to pH 8.5. Only those solutions at pH 7.0 and above killed *Bact. salmonicida* in 20 min; at pH 5.5 the bacteria survived for over 50 min.

A study was made of the action of 500 p.p.m. acriflavine solution at pH 7.7 on 23 cultures of *Bact. salmonicida*. The cultures used had been in stock in the laboratory from one week to 13 years when tested. They were isolated from 7 different epizoötics of trout furunculosis and from 4 species of trout in England and the United States. Every culture of bacterium was killed in less than 20 min. The various phases of the growth curve were also studied. Broth cultures that had been incubated from one hour to 20 days were tested, the tests being made on cultures varying by one day in age. The one-hour cultures were made from 12-day-old cultures and represented the lag phase. In no test did a culture survive longer than 20 min in

[4] Blake, Isobel, *Fisheries, Scotland, Salmon Fish*, 1930, No. 2, His Majesty's Stationery Office, Edinburgh, 1933.

[5] Browning, C. H., Gulbransen, R., and Kenneway, E. L., *J. Path. Bact.*, 1919, **23**, 106.

[6] Michaelis, L., and Hayashi, J., *Z. Immunitäts.*, 1923, **36**, 518.

[7] Eggerth, A. H., *J. Infect. Dis.*, 1926, **38**, 440.

[8] Gee, Lynn L., and Sarles, W. B., *J. Bact.*, 1942, **44**, 111.

[9] Ruehl, G. L. A., and Brewer, C. M., U. S. Dept. Agri., Circ. No. 198, 20 pp., Washington, D.C., 1931.

[10] *Manual of Methods for Pure Culture Study of Bacteria*, Biotech Publication, Geneva, N.Y., 1940.

500 p.p.m. acriflavine solution at pH 7.7.

To test the action of the disinfectant in the presence of trout eggs, ova were aseptically taken from ripe rainbow *(Salmo irideus)* and brown trout *(S. fario)*. They were charged with *Bact. salmonicida,* by placing 100 eggs in 100 ml of saline solution containing approximately 10,000,000 bacteria per milliliter. The number of bacteria adsorbed on an egg is not known, but they clouded broth medium more rapidly than eggs removed from contaminated trout abdomens. This is suggestive but is not clear cut evidence that the laboratory contaminated eggs were more heavily charged than naturally contaminated ova. Eggs were then treated for 20 min at 10° in the alkaline acriflavine solution, and removed to semi-solid, carp-infusion agar. After one week's incubation all tubes were negative for *Bact. salmonicida*. Eggs that had been exposed to the disinfectant for shorter periods carried viable *Bact. salmonicida* to the culture medium.

Next, it was desirable to know the effect of 500 p.p.m. of acriflavine at pH 7.7 on trout eggs under actual treatment conditions. Therefore, 7,500,000 ova from rainbow, brown, and brook trout *(Salvelinus fontinalis)* were treated for 30 min with the disinfectant with a total loss of only 3.65% of these eggs in the period between treatment and hatching. As a control, the records of 10,900,000 untreated eggs of the same species at the same hatcheries showed a loss of 7.92% for a similar period. Obviously, the treatment is not harmful; rather, it seems actually to benefit the eggs.

From these data, it is concluded that *Bact. salmonicida* on the shell of trout eggs is killed by a 500 p.p.m. acriflavine solution at pH 7.7 in 20 min and that the treatment of eggs for one-half hour with this solution is harmless to the eggs. Several hundred thousand trout from disinfected eggs were kept under observation for more than one year. No abnormalities in the trout were observed.

The efficacy of the disinfection in practical use cannot yet be judged because it is only one of several quarantine measures which are necessary to protect a disease-free stock. However, in one trout hatchery where egg disinfection has been combined with other quarantine measures, there has been no recurrence of trout furunculosis for 2 years.

13959

Assay of the Immunizing Effect of Scarlet Fever Toxin in the Rabbit.

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Evaluation of agents for immunization against scarlet fever has been restricted to the direct experience in human beings, the criteria of success being (1) the Dick test, (2) antitoxin determination in the serum, (3) clinical protection.[1-3] The latter will, of course, always remain the ultimate criterion of success. However, it was felt that for the development of an optimal immunizing agent for scarlet fever it would be of considerable help if a method could be found that would allow an experimental evaluation in the animal.

The assay of the scarlet fever toxin and its antitoxin in the skin of the rabbit has been successfully employed for some time.[4,5]

1 Toomey, J. A., *Ann. Int. Med.*, 1941, **15**, 959.

2 Kolchin, B. S., and Klein, J. F., *J. Immunol.*, 1941, **41**, 429.

3 Veldee, M. V., Peck, E. C., Franklin, J. P., and DuPuy, H. R., *Public Health Reports,* 1941, **56**, 957.

4 Buttle, G. A. H., and Lowdon, A. S. R., *J. Path. and Bact.*, 1935, **41**, 107.

5 Plummer, H., *Br. J. Exp. Path.*, 1934, **15**, 80.

TABLE I.
Exemplifying Dermal Resistance to Dick Toxin of Rabbits One Week After End of Immunization

Immunizing agent*	No. of rabbits treated	Pos. 1 STD	Neg. 1 STD Pos. 4 STD	Neg. 4 STD Pos. 16 STD	Neg. 16 STD Pos. 64 STD	Neg. 64 STD Pos. 264 STD	Neg. 264 STD
Scarlet Fever Toxin	13	1	0	2	3	4	3
Formalized Scarlet Fever Toxin	7	0	4	3	0	0	0
Broth	3	3	0	0	0	0	0
Not treated	5	5	0	0	0	0	0

*5 injections as described in text.

TABLE II.
Dermal Resistance to Dick Toxin of Rabbits 24 Hours After Intravenous Injection with Antitoxin.

Antitoxin	No. of rabbits injected	Pos. 1 STD	Neg. 1 STD Pos. 4 STD	Neg. 4 STD Pos. 16 STD	Neg. 16 STD Pos. 64 STD	Neg. 64 STD Pos. 256 STD	Neg. 256 STD Pos. 1064 STD	Neg. 1024 STD
Scarlatinal	10	0	0	0	4	3	2	1
Diphtheric	5	5	0	0	0	0	0	0

The following experiments will show that a dermal test in the rabbit will give an opportunity for a quantitative evaluation of both active and passive antitoxic protection.

Procedure. White rabbits were tested for sensitivity to 1 STD* of scarlet fever toxin and only such animals were employed that gave a distinct reaction. Such animals were injected with the immunizing agent, for example, scarlet fever toxin, and at the end of a period of immunization tested against the toxin. This testing was done with 1, 4, 16, 64, and 256 STD, occasionally also with 1024 STD. Appropriate controls were added. It was ascertained that healthy rabbits can be expected to stay Dick sensitive for the time period corresponding to that of immunization. In a control series, rabbits were injected with the double amount of the same nutrient broth that had been used for the preparation of the toxin. Prior experience on the Dick reaction in rabbits that had been injected with suspensions of toxigenic strains of streptococci has shown that antibacterial antibodies would not interfere.

In the first series, parallel dermal tests were made both on the ears and on the backs of each animal. The results were identical for both sites within the limit of the experimental error. In later experiments tests were made on the backs only.

Readings were made 24 and 48 hr after injection. As both readings gave similar results, only the readings after 24 hr are given in the tables.

For immunization, an erythrogenic toxin was used that was obtained according to standard methods for the preparation of toxins for human immunization. It contained 300,000 STD per ml.

A standardized toxin from the National Institute of Health was used for the dermal tests.

All dilutions were made in M/20 phosphate buffer of pH 7.2.

The animals were immunized by subcutaneous injection. We immunized several series of animals with toxin in the same 5 doses that are used for human immunization, namely: 650, 2,500, 10,000, 30,000, and 120,000 STD, the injections being spaced at 3-day intervals. One week after the last injection, the animals were tested. Typical results of such tests can be seen from Table I. The individual response varies considerably.

* STD — Skin Test Dose. For definition see f. i.: A. B. Wadsworth, *Standard Methods of the Division of Laboratories and Research of the New York State Department of Health*, Second Edition, Baltimore, 1939, Williams & Wilkins Co., p. 382.

"Failure to respond to 100 STD" would indicate the order of average resistance acquired under the conditions of the procedure of immunization just described.

Parallel with some of these series, animals were injected with the same toxin treated with 0.3% formol until well over 90% of its toxicity as measured in the rabbit skin had disappeared. Some immunizing effect was observed (Table I), but markedly less than with the original toxin. It is irrelevant for the moment whether this remaining effect was due to the traces of unmodified toxin still present. The purpose of reporting these experiments here is to show that quantitative differences in immunizing effect can be detected by the intradermal test.

We recommend to test the acquired resistance one week after completion of immunization. At this time, dermal resistance is at its high point. Animals tested 2 weeks after completion of immunization already showed signs of lessening of resistance.

As a preliminary standard for comparative experiments the immunizing effect of scarlet fever toxin in amounts as used for human immunization might form a useful basis. It is obvious from the tables that 6 to 10 animals should be employed in each series to guard against deception by individual variation.

Dermal resistance to toxin can also be obtained by passive immunization. Two groups of rabbits were injected with commercial scarlatinal antitoxin (Globulin Modified, Lederle), in a dosage of 1 U.S.P.H.S. unit per g body weight. At the same time, rabbits were injected with the same amount (measured as serum protein) of commercial diphtheric antitoxin (Globulin Modified). Twenty-four hr later, all animals were tested with graded amounts of toxin in the skin. Results can be seen from Table II. It will be seen that a dermal resistance similar in degree to that obtained with active immunization with scarlet fever toxin was obtained after passive immunization with scarlatinal antitoxin, whereas injection with diphtheric antitoxin would not change Dick sensitivity.

Summary. Dermal resistance to graded amounts of Dick toxin can be used as a method for evaluation of agents for scarlet fever immunization.

13960

Simplified Technic for Inoculating into Amniotic Sac of Chick Embryos.*

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Burnet[1] has reported the isolation of influenza virus direct from throat washings, by inoculation into the amniotic sac of chick embryos and subsequent passages with a suspension of the ground tracheas removed from the embryos following a suitable period of incubation.

The technic employed by Burnet involves cutting a window in the egg shell over the embryo, puncturing the shell membrane and transferring the air sac to between the shell and the chorioallantoic membranes beneath the opening. The cut portion of the shell, including the attached shell membrane, is then removed exposing the chorioallantois beneath the window in the shell. A small slit is made in the chorioallantois, and by means of a fine forceps the underlying amniotic membrane is grasped and drawn through the opening in the chorioallantois. Inoculation is made into the amniotic sac by puncturing the exposed membrane with a capillary pipette. The membrane is then released and allowed to withdraw through the opening in the chorioallantois. Finally, the window in

* These studies were supported in part by a grant from the International Health Division of The Rockefeller Foundation.

1 Burnet, F. M., *The British J. Exp. Path.*, 1940, **21**, 147.

the shell is closed with a cover glass and the edges sealed with paraffin. Unless a very large opening is made in the shell at the time of the inoculation, it is necessary to enlarge it further, following incubation, for the removal of the embryo.

Experimental. We have found the following technic to be somewhat simpler than the one described by Burnet.

During transillumination of the egg to determine whether the embryo is alive, the border of the air sac is marked upon the shell with a pencil. The shell under the circular pencil mark is then cut through with a dental drill, leaving the shell membrane intact. Alcohol is applied, the exposed shell membrane is cut through with sterile scissors,

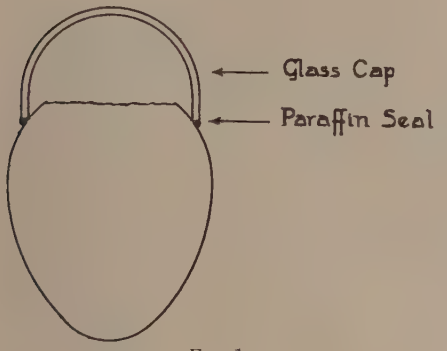

FIG. 1.

and the shell cap over the air sac is removed, thus exposing the diverted layer of the shell membrane which covers the chorioallantois. While dry the exposed membrane is quite opaque, but if a drop or two of 50% alcohol is placed upon it, it becomes sufficiently translucent to make visible the border of the yolk sac and the larger blood vessels in the chorioallantois. Avoiding the yolk sac and the blood vessels, the closed points of a fine curved forceps are thrust through the outer membrane and chorioallantois. The forceps are then slightly opened, and the underlying amniotic sac is grasped and pulled through the chorioallantois and outer membrane. While the tent-like fold of the amniotic membrane is being held by the forceps, inoculation is made into the base of the exposed sac with a tuberculin syringe attached to a fine needle (27 gauge). The fold of the membrane is released and of its own accord slips back into place.

The circular opening is then covered by an inverted glass cup with an inside diameter of 32 mm (Fig. 1). In order to obtain a hermetic seal the brim of the cup is dipped in melted paraffin (melting point 45°) just before placing it upon the egg. The eggs are incubated in an upright position (cup end upward).

When it is desired to examine the embryo following incubation, the cup is removed, the shell membrane and the chorioallantois are cut away, and the embryo is lifted out with a pair of forceps and placed in a Petri dish. No further enlargement of the opening in the shell is required.

If it is desired to examine the allantoic fluid, this is first withdrawn by means of a 10 cc syringe with a 22 gauge needle. The needle is thrust through the shell and chorioallantoic membranes near the side of the shell, and the fluid is aspirated. It is usually found to be clear and free from red blood cells. Following withdrawal of the allantoic fluid, the membranes are cut away with scissors thus exposing the embryo and the amniotic sac. The amniotic fluid may be obtained by either puncturing the sac *in situ*, or after it is removed with the embryo.

Occasionally an egg is encountered (not more than one in 10) in which the yolk sac almost completely covers the under surface of the air sac. In such an event some difficulty may be experienced in seizing the amniotic sac and drawing it to the surface, but this can usually be accomplished.

In our experience the advantages of this method are: (1) it is not necessary to determine in advance the exact location of the embryo. (2) The shell can be cut about the natural air sac with greater facility than over the embryo, as there is no danger of injuring the blood vessels of the chorioallantois. (3) It does not require the transference of the air sac. (4) The allantoic liquid may be easily removed free from blood cells. (5) By means of an inverted glass cup the opening in the shell can be easily and hermetically

sealed. (6) No further enlargement in the opening in the shell is required for removal of the embryo.

Using this technic of inoculating eggs, we have succeeded in isolating influenza virus A from throat washings which had been preserved at a temperature of approximately —76° for nearly 2 years.

13961

Influence of Estradiol Benzoate on Fat Storage.*

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In a recent study Loeb and Burr[1] showed that female rats store more body fat than males when maintained on a diet rich in saturated fat (hydrogenated coconut oil) but devoid of essential fatty acids. It then became a matter of interest to ascertain whether this finding could be attributed to the effect of estrogen on fat metabolism. Zondek and Marx[2] reported that estrogen not only induced a marked lipemia in fowl but also caused the accumulation of fat in the internal organs, particularly the liver. However, their findings with mammals were negative. Loeb[3] observed a definite, though moderate, lipemia in male rats fed a high fat diet lacking in essential fatty acids when estradiol benzoate was injected over a 4-week period. The present report is concerned with the storage of body fat under similar conditions.

Methods. Albino rats of the Wistar strain were taken at weaning time and placed on a low fat diet for 4 weeks to deplete the fat reserve. During the next 8 weeks, which comprised the experimental period, the animals were maintained on diet 580-B, a ration rich in saturated fat—71% hydrogenated coconut oil. One group received, subcutaneously, 3 large doses of estradiol benzoate‡ (100 μg per dose§), on alternate days, during the last week of the experimental period. Two other groups were given 24 more moderate doses (30 μg and 5 μg, respectively), daily except Sunday, throughout the last 4 weeks of the experimental period.[3] The animals were sacrificed by etherization and the carcasses† analyzed for total fat by digestion with 28% KOH in 50% alcohol for 4 hr followed by petroleum ether extraction of acidified aliquot portions. All analyses were done on duplicate aliquot portions which checked well within 1%.

Table I shows the mean values for total carcass lipid of the groups investigated. It will be seen that female controls (received no estradiol benzoate) had an appreciably higher carcass fat than the males. Rats which received 3 large doses of the hormone during the last week had a lower fat content than their male controls; but this effect was due to diminished food consumption under the influence of large doses of estrogen. Group C showed a higher content of body

* Assistance in the preparation of these materials was furnished by the personnel of Work Projects Administration, Official Project No. 265-1-71-236, Sub-project No. 382.

† Rockefeller Foundation Fellow.

1 Loeb, H. G., and Burr, G. O., unpublished results.

2 Zondek, B., and Marx, L., *Arch. Int. Pharmacodyn.*, 1939, **61**, 77.

3 Loeb, H. G., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 340.

4 Fisher, R. A., *Statistical Methods for Research Workers*, 7th Ed., Oliver and Boyd, London, 1938.

‡ The author wishes to thank Dr. R. G. Floody (Roche-Organon, Inc.) who kindly supplied the estradiol benzoate.

§ Each dose in every case was contained in 0.05 cc of olive oil.

† The carcass was prepared for analysis by removing the head, tail, and limbs.

TABLE I.
Carcass Fat of Rats Receiving Diet 580-B and Estradiol Benzoate.

Group	No. of rats	Total estradiol benzoate (μg)	Total lipid, %	Groups compared	t*
A ♂	6	—	13.0 ± 0.93†	A vs. B	4.32
B ♀	6	—	20.1 ± 1.13		
C ♂	5	720 (24)	16.1 ± 0.87	A vs. C	2.16
D ♂	6	120 (24)	12.7 ± 0.37		

*The t value was employed according to Fisher;[4] for the 5% level the value is 2.2, and 3.0 for the 1% level.
†Standard error of the mean.

TABLE II.
Food Consumption of Rats Receiving Estradiol Benzoate and Controls.

Group*	Food intake (2nd to 7th wk., incl.) g/rat/day	Food intake (2nd to 7th wk., incl.) cal/rat/day	Food intake (2nd to 4th wk., incl.) g/rat/day	Food intake (5th to 7th wk., incl.) g/rat/day
580-B Controls				
A1 ♂	3.02	22.1	2.79	3.24
A2 ♂	3.44	25.2	3.14	3.73
B1 ♀	3.07	22.5	2.73	3.41
B2 ♀	3.21	23.5	3.14	3.27
580-B M.E.†				
C1 ♂	3.67	26.9	3.62	3.73
C2 ♂	3.33	24.4	3.48	3.19
580-B L.E.†				
D1 ♂	3.67	26.9	3.56	3.79
D2 ♂	3.92	28.7	4.00	3.84

*Each of the 8 groups contained 3 rats except for Group C2 which had 2.
†M.E. = Medium estrogen dose (24 30-μg injections during 5th to 8th weeks, inclusive).
L.E. = Low estrogen dose (24 5-μg injections during 5th to 8th weeks, inclusive).

fat while no change was observed in Group D.

In evaluating the data it is necessary to take into account the effect of estrogen administration on appetite. Table II shows the average food consumption for 6 of the 8-week experimental period, and also for the 3-week period prior to hormone administration and the 3-week period during estrogen administration. Comparing Groups A and B it is found that the increased fat storage in females can not be ascribed to an increased food intake. The figures for Groups C and D show that administration of estradiol benzoate had no significant influence on appetite. The increased food consumption of rats in Group C as compared with the controls would not explain the increased fat storage since individuals of Group D which consumed as much or more food than those of Group C stored less fat. We are led to conclude that estradiol benzoate, *per se*, when given daily at a level of 30 μg over a 4-week period causes an increase in body fat.

From Table I it is apparent that the difference in fat storage of males and females when no estrogen is administered is highly significant. The increased body fat of rats receiving 30 μg doses of the estrogen can not be explained by increased food intake, and the t value indicates that the difference is significant. The smallest dose administered (5 μg) did not influence the storage of body fat although, as shown in an earlier study, it did induce a moderate lipemia.

Turner and Mulliken[5] have recently reported that testosterone propionate administration accelerates the removal of corn oil injected subcutaneously in mice. With the large doses of estradiol benzoate which they

[5] Turner, J. C., and Mulliken, B., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 585.

employed (300 μg in 2 doses) about half of their animals died and the effect on injected oil was inconclusive in the remaining individuals. Since estradiol benzoate at a 30 μg level (24 injections) brought about an increase in carcass fat it is possible that the ratio of androgens to estrogens determines the net effect of these hormones on body fat.

Summary. Estradiol benzoate, *per se,* causes an increased storage of body fat in male rats when they are maintained on a diet rich in saturated fat but devoid of essential fatty acids. The effect depends on the size of the dose and the length of the period in which it is administered.

13962 P

Electron Micrography of the Eastern Strain Equine Encephalomyelitis Virus.

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In recent reports from this laboratory, there have been described the results of preliminary studies with the electron microscope on two small animal viruses, namely, the agent of rabbit papillomatosis[1] and that of the Western strain equine encephalomyelitis.[2] The present paper is concerned with the findings in a similar examination of the Eastern strain equine encephalomyelitis virus.

The virus was propagated in chick embryos and purified by ultracentrifugation as previously described.[3] The final product was dissolved in Ringer solution[4] pH 8.5. Examinations have been made of the virus obtained from 5 different batches of diseased embryos at various intervals after purification. The dried films for electron micrography were prepared as previously described.[1,2]

A micrograph of the Eastern strain equine encephalomyelitis virus is shown in Fig. 1. For preparation of the film a solution containing 2.2 mg virus per cc in Ringer fluid was diluted fourfold with distilled water. As shown, the predominant images are circular in shape and appear as individuals or small irregular agglomerates dispersed at random over the field. There is present also a small amount of ill-defined amorphous extraneous material. The circular images are, like those of the papilloma and Western strain viruses, uniform in size, shape and appearance. The central dark area of the images indicates, through greater scattering of electrons, a relatively high density of the corresponding region of the virus particle. Extending peripherally from the central area, the enveloping substance appears to be progressively less dense and finally fades indefinitely into the film background. This appearance of the generality of the images gives the impression to be expected of a spherical particle made up of a loose structure of regularly arranged diffraction centers, the density in the thickest dimension diminishing toward the outer limits. Evidence of structure and differentiation internally was not generally observed in fresh preparations.

* This work has been aided by grants from Lederle Laboratories, Inc., Pearl River, New York, and by the Dorothy Beard Research Fund.

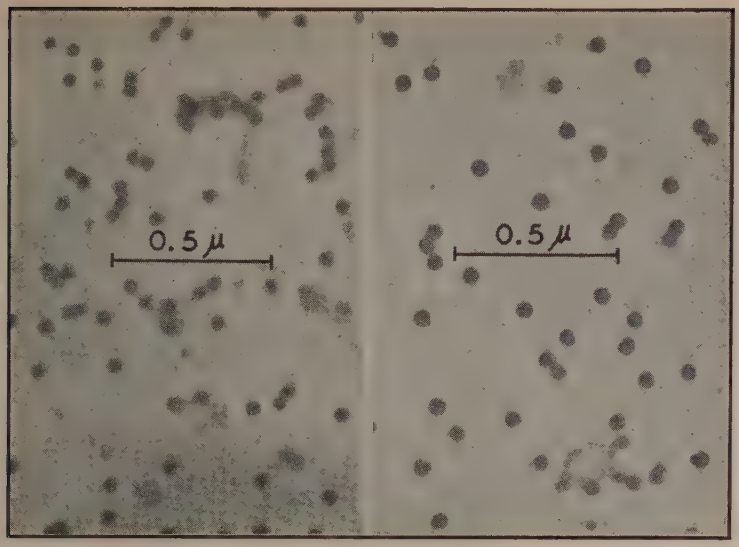
1 Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 205.

2 Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 206.

3 Taylor, A. R., Sharp, D. G., Beard, D., and Beard, J. W., *J. Infect. Dis.*, in press.

4 Bayliss, W. M., *Principles of General Physiology*, Longmans, Green and Co., Ltd., London, 4th ed., page 211.

5 Stanley, W. M., and Anderson, T. F., *J. Biol. Chem.*, 1941, **139**, 325.



Equine Encephalomyelitis Virus, Eastern Strain.

FIG. 1. Virus particles placed on collodion film in Ringer solution diluted 1:4 with water.

FIG. 2. Virus particles in Ringer solution diluted 1:3 with 0.25% $CaCl_2$ solution and kept at room temperature for 1 hour before preparation of film. Photographs were taken with 55 kilovolts electrons. The magnification is 46,000 ×, calculated from measurements on the width of tobacco mosaic virus rods which has been reported to be 15 mμ.[5]

In some of the images, however, there appeared to be a definite central rounded area of increased density approximately two-thirds of the total image diameter.

The vagueness of external outline of the images seen in Fig. 1 has been a regular observation with the Eastern strain virus, and, too, with the papilloma virus[1] and with fresh preparations of the Western strain equine encephalomyelitis virus.[2] In the case of the Western strain virus, the images were invariably more clearly defined after the virus had stood some days in Ringer solution at 2 to 8°C. On the hypothesis that the particle density with respect to electron scattering might be associated with salt factors, studies were made to learn the effect of changing the concentration and proportion of the salts of Ringer fluid. No definite effect was observed with NaCl or KCl. On the other hand, the character of the images was markedly altered with changes in $CaCl_2$ concentration. In Fig. 2 is shown the micrograph of a virus preparation in Ringer fluid diluted threefold with 0.25% $CaCl_2$ and kept for 1 hr at room temperature before preparation of the film. No attempt was made to wash the prepared film. The images of highly uniform size now show clearly defined limits with a reversal of the regions of relative density. In the central area, the density appears less than that toward the surface, revealing a relatively light (in the micrograph) structure corresponding in position to the rather indefinite dense internal region of the images of Fig. 1. This increase in density related to $CaCl_2$ was reversible, at least in great part, since washing with water of a film prepared as for Fig. 2 resulted in a micrograph like that of Fig. 1. Treatment of the virus with $MgCl_2$ produced an effect somewhat similar to that seen with $CaCl_2$.

The average diameter of the particles of Fig. 1, including the outermost vague limits was 40.2 mμ as compared with 47.5 mμ for the particles of Fig. 2. The mechanism responsible for the alteration in the appearance

and size of the images of the virus particles is not clear. There is the possibility that the calcium salt may have entered into a loose combination with the peripheral substance, which, by this means, became sufficiently dense for demonstration. The apparently greater size of the particles represented in Fig. 2, however, was not paralleled by change in the sedimentation rate which was not altered by treatment for 1 hr with $CaCl_2$. It is unlikely that the change was due wholly to drying effects occurring at the particle surface while on the film, since the appearance of the image was altered only when the virus was in contact with the $CaCl_2$ for some time in solution.

The present results indicate that the Eastern strain equine encephalomyelitis virus is probably spherical in shape, constituted peripherally of a substance the limits of which were ill-defined in the micrographs in the absence of special treatment. The average diameter of the particles of vague outline of Fig. 1 was 40.2 mμ. If the true particle limits are those portrayed after treatment with $CaCl_2$, the diameter was 47.5 mμ.

13963

Further Experiments Dealing with Embryonic Enucleation in Amblystoma.

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In a previous communication[1] we described the results of embryonic enucleation in *Amblystoma punctatum* upon growth, metamorphosis and pigmentary changes. Our results upon growth did not conform with those of Browman[2] who found that rats enucleated at birth, regardless of environment, had a growth rate consistently lower than their litter mate controls of the same sex.

Browman[3] found that rats enucleated at birth suffered retarded development of the genital tracts and the gonads. We investigated this matter in *Amblystoma punctatum,* but could find no evidence at metamorphosis because of insufficient development of the gonads. Because of the difficulties of raising *A. punctatum* through metamorphosis, we have repeated the work on *A. tigrinum* and *A. mexicanum* (axolotl).

Twenty embryos (tail bud stage) of *A. tigrinum* were enucleated. Ten eyeless and 5 normal controls were reared in darkness, and 10 eyeless with 5 controls were kept under ordinary daylight illumination. Ten unilateral and 10 bilateral enucleations were done on axolotl embryos (tail bud stage). These along with 10 normal embryos were reared in aquaria under normal conditions of illumination.

In *A. tigrinum* we dissected the gonads of 5 females without eyes and 2 with eyes which had been raised in the dark; also 3 males without eyes and 3 with eyes reared in the light. The animals studied ranged from 109 to 170 days of age. All had metamorphosed except one. We found considerable variation in the size of the ovaries and the testes irrespective of the presence or absence of eyes or the conditions of illumination. Although the gonads were well differentiated, they had not reached maturity in either the experimental or the control animals. Because of our failure to obtain evidence of any effects of eyelessness upon gonadal development in *A. tigrinum* up to 170 days of age, we reared the axolotls *(A. mexicanum)* for two years. At the end of this period, we dissected 2 control and 5 eyeless axolotls and found no significant size difference between the gonads of the two groups.

[1] Detwiler, S. R., and Copenhaver, W. M., *Anat. Rec.,* 1940, **76**, 241.

[2] Browman, L. G., *Anat. Rec.,* 1938, **72**, Suppl. 41.

[3] Browman, L. G., *Anat. Rec.,* 1938, **72**, Suppl. 122.

Our observations showed that there was no difference in the growth rate between eyeless and normal forms in either species studied. Throughout the entire period, eyeless axolotls, reared in the light, were pale; normals were decidedly dark. The one-eyed axolotls, with respect to color changes, behaved like the normal 2-eyed animals. This situation agrees with the findings of Parker[4] on Fundulus and is in contrast with his findings on Ameiurus.

Pale eyeless axolotls 9 months old when injected with intermedin,* became dark but never as dark as the normal 2-eyed controls. The effect wore off gradually and at the end of a week the original light color was restored.

Removal of the second eye from a one-eyed axolotl at 9 months, caused the animal to become pale, but not as pale as those rendered eyeless in the embryonic stage. Enucleation of a 12-year-old axolotl had no effect upon coloration. The animal remained in a permanently dark state.

Summary. Embryonic enucleation of *A. punctatum, A. tigrinum* and *A. mexicanum* (axolotl) has no significant effect upon the growth rate. The influence of the eyes upon color changes and possibly upon metamorphosis seems not to be related to any eye substance (hormone) developed within the eye itself.[1] No effect of embryonic enucleation upon the development of the gonads could be found in *A. tigrinum* up to 170 days after the embryonic stage nor in axolotls two years of age.

[4] Parker, G. H., *Proc. Nat. Acad. Sci.*, 1939, **10**, 499.

* The preparation which we used was made from the anterior lobe of the whale, and was kindly supplied by Dr. Geiling of the University of Chicago.

13964

Serial Bone Marrow Studies in Pernicious Anemia. I. Fluctuation in Number and Volume of Nucleated Cells.

JOSEPH STASNEY AND PHILIP PIZZOLATO.

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A number of authors[1–14] who studied the bone marrow at intervals after the beginning of liver-induced remission emphasized the rapid disappearance of megaloblasts and the appearance of normoblasts. These authors obtained by biopsy[1,2,7] or aspiration technic[3–5, 7–14] specimens from patients with pernicious anemia before liver treatment and one or two specimens at varying intervals during liver therapy. Some authors aspirated the same patient 2 or 3 times at different intervals during liver therapy,[5,12,14] or in groups of from 4 to 10 patients, performing the aspiration in different phases of their re-

[1] Zadek, I., *Klin. Wchnschr.*, 1924, **3**, 1483.

[2] Peabody, F. W., *Am. J. Path.*, 1927, **3**, 179.

[3] Tempka, T., and Braun, B., *Folia Haemat.*, 1932, **48**, 355.

[4] Segerdahl, E., *Acta Med. Scand.*, Suppl., 1935, **64**, 1.

[5] Nordenson, N. G., *Studies on Bone Marrow from Sternal Puncture*, Stockholm, Börtzells, Esselte, 1935.

[6] Isaacs, R., *Am. J. Med. Sc.*, 1937, **193**, 181.

[7] Dameshek, W., and Valentine, E. H., *Arch. Path.*, 1937, **23**, 159.

[8] Storti, E., *Hematologica I Archivio*, 1937, **18**, 1.

[9] Schartum-Hansen, H., *Folia Haemat.*, 1937, **58**, 145.

[10] Koller, F., *Deutsches Arch. f. Klin. Med.*, 1939, **184**, 568.

[11] Bock, H. E., and Malamos, B., *Folia Haemat.*, 1939, **62**, 408.

[12] Rohr, K., *Das menschliche Knochenmark*, Georg Thieme, Leipzig, 1940.

[13] Houghton, B. C., and Doan, C. A., *Am. J. Clin. Path.*, 1941, **11**, 144.

[14] Davidson, L. S. P., Davis, L. J., and Innes, J., *Quart. J. Med.*, 1942, **11**, 19.

mission.[4,5] In one study[11] the marrow of one patient had been studied 17 times at frequent intervals and in other studies[5,8,13] the aspiration was performed at regular 48-hr intervals 5 or 6 times. These authors studied the morphological and proportional changes of the different types of cells. Only 3 reports have included quantitative data.[4,6,9] One study concerned cases of pernicious anemia on which bone marrow studies were performed at necropsy. These patients died at varying intervals after the institution of liver therapy.[6] More recently 2 authors attempted to follow the bone marrow changes by frequent serial marrow punctures. Their studies included both qualitative and quantitative data.[4,9]

In the following study, an attempt was made to follow the quantitative cellular changes of the sternal marrow during liver-induced remission.

Materials. In this study 16 patients with Addisonian pernicious anemia were observed. These cases were divided into 3 groups according to the dose of liver extract administered during the first 10 days of hospitalization. The first group included 9 patients receiving more than 60 USP units. The second group included 5 patients, 4 of whom received 30 units and the fifth, 40 units. The third group included 2 patients, each of whom received one unit daily.

Technic. The marrow samples from the same patient were obtained on one or 2 occasions before therapy had been instituted and then at 24-hr intervals. In 3 instances the first puncture was obtained 6 hr, in one 9 hr, and in two 12 hr after the first liver injection.

The subsequent aspirations were performed at about 24-hr intervals for an average of 9 times. Different portions of the sternum were aspirated with a 17-18 gauge needle, and one cc of marrow was obtained. The total nucleated elements and reticulocyte counts were performed instantly after aspiration of the marrow. For the total nucleated elements the marrow was diluted 1:20 with 1% acetic acid using the standard white cell pipette and counting all nucleated elements in 80 small squares under high dry objective, and to the number obtained 3 ciphers were added. This gave the number of nucleated cells of all types per cubic mm of aspirated marrow. The volume percentage of the total nucleated elements together with the volumes of fat, plasma and erythrocytes were determined according to the Limarzi technic,[15] using as an anticoagulant a dried mixture of ammonium and potassium oxalates. Smears for the differential count were made from the mixed buffy coat after centrifugalization. Five hundred cells were counted and the percentage of megaloblasts, pronormoblasts, normoblasts and other myeloid cells was computed.

Daily peripheral blood studies on oxalated blood included the determination of hemoglobin (Hellige), the number of red and white blood cells, hematocrit (Wintrobe), and percentage of reticulocytes, using brilliant cresyl blue counterstained with Wright's stain.

To ascertain the homogeneity of different portions of the sternum, the marrow was aspirated from two sites 5 cm. apart in 7 control patients. These double marrow punctures were repeated at 24-hr intervals using again different sites.

Observations. Preliminary studies revealed that marrow samples taken simultaneously at 2 different sites of the sternum exhibited comparable quantities of nucleated cells of all types. We found also that the number of cells varied proportionately with the amount of the buffy coat. The normal value of these cells ranged between 30,000 and 50,000 per mm^3 while the volume of buffy coat ranged between 3 and 8%. These two data together usually reflected quite satisfactorily the functional status of the marrow.

Table I illustrates marrow samples obtained from 16 patients in relapse. Twelve samples exhibited hyperactivity, and 4 were within normal limits, as judged by the number of cells. Twenty-four hr after the beginning of liver treatment, there was a drop in the number of nucleated cells in 13 instances. In 2 instances there was a marked rise in the number of nucleated cells which

[15] Limarzi, L. R., *Illinois Med. J.*, 1939, **75**, 38.

TABLE I.

Shows the Fluctuation in the Total Number of Nucleated Marrow Cells Following the Injection of Liver Extract in 16 Patients.

Case No.	Red blood cells*	Liver dose†	Nucleated marrow cells in thousands: Before therapy	Hr after 1st dose: 6-24	48	72	Fluctuation: Min.	Max.	Time and peak of blood reticulocytes: Hr	%
1	2.00	60	268	160	197	45	20	197	72	24.6
2	1.30	75	250	121	66	68	10	157	120	20.0
3	0.75	210	145	40	41	225	38	245	—	—
4	0.77	315	93	298	48	357	48	357	120	40.0
5	1.70	180	107	222	129	239	15	239	144	30.4
6	0.76	180	40	38	40	73	38	129	144	30.0
7	1.15	135	176	35	87	165	35	165	96	35.0
8	1.70	100	162	72	98	140	5	140	120	16.0
9	1.80	60	185	136	109	100	30	136	120	28.0
10	1.20	40	202	107	267	282	17	282	120	27.0
11	1.30	30	170	53	201	117	12	201	96	32.8
12	1.50	30	49	105	40	—	40	105	96	33.0
13	1.00	30	112	92	114	63	31	114	96	37.0
14	1.70	30	42	19	21	105	18	105	96	39.0
15	1.30	10	159	99	212	38	7	256	144	26.0
16	1.70	10	32	31	37	60	31	60	144	18.0

*In millions.
†U.S.P. units.

did not decrease until 72 hr later. In one instance there was no change in the number of cells. Within 48 to 72 hr after the first administration of the liver extract, there was, in all cases, a periodic fluctuation in the number and volume of nucleated cells. The majority of the cases showed 3 irregular fluctuations in cellularity during the 10-day period following the institution of liver therapy. This was followed by a period in which more constant numerical data were obtained.

These changes in the total number of nucleated cells presented a marked individual variation. The extent of the fluctuations was independent of the amount of liver administered. No direct correlation existed between the degree of fluctuation and the number of reticulocytes. An overdose of liver did not accelerate the maximum response of the reticulocytes.

The morphological study of these marrow samples revealed a rapid disappearance of the megaloblasts within 24 to 48 hr and the presence of an increased number of early normoblasts. Further analysis showed that the fluctuations of the nucleated elements were almost entirely due to the periodic increase and decrease in the number of these cells of the normoblastic series (Fig. 1). The increase of reticulocytes in the marrow preceded or was simultaneous with the increase of reticulocytes in the peripheral blood.

Comment. In this study an attempt was made to distinguish between the megaloblast and pronormoblast only on the basis of the chromatin distribution in their nuclei and the degree of basophilia of their cytoplasm.[16] A rapid increase of basophilic cells with a tendency for chromatin condensation in their nuclei was noted, and was interpreted as normoblastic stimulation. These latter cells frequently showed mitotic and amitotic forms. The prompt decrease of cellularity soon after the beginning of liver administration in the majority of the cases suggested a "purging" process of megaloblasts in the marrow while the subsequent increase in cellularity indicated an erythroid stimulation.

The specimens obtained during the period from 96 to 168 hr after the administration of

[16] Jones, O. P., *Cytology of Pathologic Marrow Cells with Special Reference to Bone-Marrow Biopsies.* Downey, H., *Handbook of Hematology,* Hoeber, New York, 1938, 3, 2045.

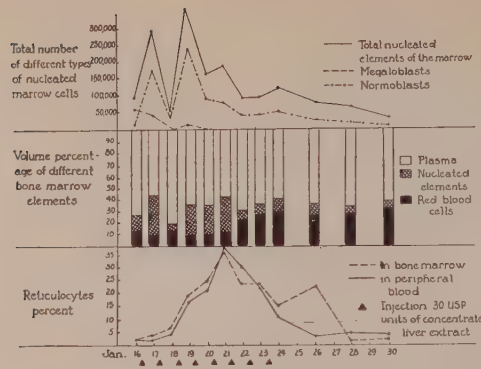

FIG. 1.

Illustrates the changes in the number and volume of the nucleated elements of the marrow following large doses of liver extract.

the first liver dose showed again a marked decrease in the number and volume of the total nucleated cells which was due to an almost complete depletion of the normoblastic cells in the marrow. This was again followed by marked normoblastic proliferation.

With these data we confirmed and elaborated the observations made by others using a similar technic.[4,8,9] These authors also noticed a fluctuation of the total number of the nucleated marrow cells.

Summary. Serial sternal marrow studies at 24-hr intervals before and after liver induced remission in 16 patients with Addisonian pernicious anemia, revealed a marked fluctuation of the total number and volume of the nucleated elements. Six to 24 hr after the first injection of liver the majority of the cases showed a marked decrease in the total number of nucleated cells. This was followed by a periodic increase and decrease of the absolute number of the normoblasts. During the period from 96 to 168 hr after the institution of liver therapy there was at times an almost complete depletion in the marrow of these normoblasts.

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Serial Bone Marrow Studies in Pernicious Anemia. II. Nucleated Cells and Blood and Urine Uric Acid.

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Because of an increase of the uric acid levels in the blood in pernicious anemia, early observers assumed that a primary purine disturbance was present in this disease.[1-3] Increased amounts of uric acid in the blood and urine simultaneous with a rise in the blood reticulocytes has been observed in pernicious anemia during liver induced remission,[4,5] in iron deficiency anemias,[5] and in experimentally produced anemias.[6,7] Few authors reported acute attacks of gout following liver administration in patients with pernicious anemia.[8-10] The increased amount of uric acid in these conditions was regarded as a disintegration product of the nucleic acid portion of the extruded nuclei of the normoblasts.

In the previous communication there was evidence of marked fluctuation in the number of nucleated marrow cells during the early phases of liver induced remission. This fluctuation was due to irregularly

1 Rosenquist, E., *Z. f. klin. Med.*, 1903, **49**, 193.

2 Gettler, A. O., and Lindeman, E., *Arch. Int. Med.*, 1920, **26**, 453.

3 Gibson, R. B., and Howard, C. P., *Arch. Int. Med.*, 1923, **32**, 1.

4 Riddle, M. C., *J. Clin. Invest.*, 1929-30, **8**, 69.

5 Cotti, L., and Balestrieri, F., *Gior. di clin. med.*, 1939, **20**, 628.

6 Krafka, J., Jr., *J. Biol. Chem.*, 1929, **83**, 409.

7 Krafka, J., Jr., *J. Biol. Chem.*, 1930, **86**, 223.

8 Sears, G. W., *Lancet*, 1933, **224**, 24.

9 Opsahl, R., *Acta Med. Scand.*, 1939, **102**, 611.

10 Broechner-Mortensen, K., *Medicine*, 1940, **19**, 161.

periodic proliferation and depletion of the normoblasts in the bone marrow. In the present study an attempt has been made to trace these normoblasts disappearing from the bone marrow by finding a concomitant increase of the uric acid level in the blood and urine.

Material. In patients with Addisonian pernicious anemia, in addition to the previously detailed bone marrow and blood studies, daily uric acid of the blood was determined in 15 cases, using the method of Folin.[11] The amount of uric acid of the

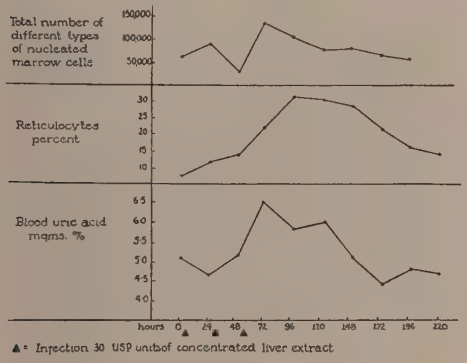

FIG. 1.

A composite chart of 7 cases illustrates that the depletion of nucleated cells in the marrow precedes the rise in reticulocytes and in the level of the uric acid of the blood.

24-hr urine was also determined in 3 of these cases.[11] Three of the patients were on a purine poor diet over a period of 20 days while the others were on the regular hospital diet.

Observations. With the above technic, 2-4 mg % was considered the normal range of the uric acid level in the blood. There were 24 observations on patients with pernicious anemia in relapse. In 8 instances the values were from 4.2 to 8.9 mg %, giving an average of 5.28 mg %; in two instances the values were 1.4 and 1.6 mg %.

Fig. 1, a composite graph of 7 cases, illustrates that when the bone marrow showed a depletion of the nucleated elements, there was a concomitant increase in the amount of uric acid in the blood preceding but roughly paralleling the rise in the reticulocytes.

[11] Folin, O., *J. Biol. Chem.*, 1933, **101**, 111.

These cases, with an average of uric acid in the blood already above normal, showed more elevated levels varying from 5.3 to 10 mg % within 72-120 hr after the first liver extract injection. In 9 instances the peak of the uric acid in the blood was 24-48 hr prior to, and in 5 instances, it was simultaneous with, the peak of the reticulocytes.

Fig. 2 illustrates a typical response of a patient on a purine poor diet for 20 days who received 30 U.S.P. units of liver extract. It shows that when there was a fall in the total nucleated elements in the bone marrow there was a simultaneous rise in the blood reticulo-

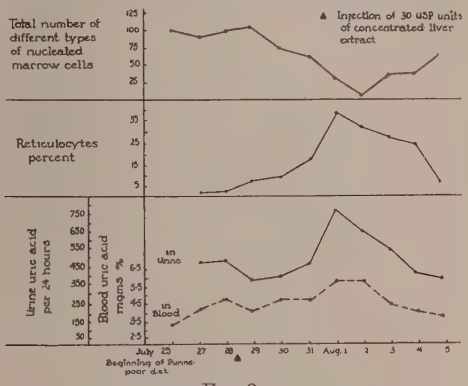

FIG. 2.

Illustrates the changes in the total number of nucleated marrow cells, reticulocyte percentage of the blood, and the level of the uric acid of the urine and blood, following the administration of 30 USP units of liver extract in a patient with pernicious anemia.

cytes and in the level of the uric acid of the blood and urine.

In 14 patients, during the period of observation from 12-30 days, there was no significant elevation in the uric acid level of the blood other than that corresponding to the rise of the reticulocytes. In 2 cases of macrocytic hyperchromic type of anemia, which did not respond to liver injection with a marked increase of reticulocytes, there was no increase in the uric acid level.

Summary. There was a definite increase of the uric acid level in the blood and urine simultaneous with the diminution of the normoblasts in the bone marrow and with the increase of reticulocytes in the peripheral blood.

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Serial Bone Marrow Studies in Pernicious Anemia. III. Occurrence of Protoporphyrin in Human Bone Marrow.

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The occurrence of preëxisting protoporphyrin in red blood cells of the peripheral blood[1,2] and in the megaloblasts and erythroblasts of the bone marrow has been demonstrated.[3] In the normal blood red fluorescing erythrocytes, which were called "Fluorescyten," were reported.[4] These cells became increased in proportion to the erythrocytic regeneration.[5] They were found to be numerous in the blood of patients with pernicious anemia during their response to liver therapy.[6] Subsequently it was shown by chemical methods that the increase of protoporphyrin in the blood was simultaneous with the increase in the number of "Fluorescyten."[7,8] Later it was concluded that the "Fluorescyten" were younger red blood cells,[9] and that the increase of protoporphyrin concentration of the blood was directly proportional with the number of reticulocytes present.[10,11] An increased protoporphyrin content of the bone marrow was found following hemorrhagic anemia in rabbits.[12]

In the preceding communications, data were presented showing that during the early phases of liver induced remission in the bone marrow of patients wtih pernicious anemia there was a marked normoblastic stimulation. The rapidity of morphological and quantitative changes which are characteristic of the normoblastic transformation of a megaloblastic marrow is suggestive of a change in cellular chemistry, which might be manifested also in variations of the protoporphyrin content. This assumption has prompted the present investigation.

Materials and Methods. One hundred and thirty-three bone marrow specimens were examined. Ninety-six marrow specimens were obtained from 12 patients with Addisonian pernicious anemia before and at frequent intervals during their response to liver therapy. Thirty-seven additional marrow specimens were obtained from patients with iron deficiency anemias, posthemorrhagic anemias, hemolytic anemias, sprue and leucemias.

The technic for obtaining marrow samples and for the determination of the total nucleated elements and of the percentage of reticulocytes has been described in detail in the preceding communication.

The following technic was used for the determination of the presence of protoporphyrins:

About 0.4-0.5 cc of the fresh sample of washed and packed bone marrow cells was placed in a 15 cc centrifuge tube. Ten cc of a mixture of 1 part glacial acetic acid and 2 parts of ethyl acetate were added. The

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5 Seggel, K. A., *Folia Haemat.*, 1935-36, **54**, 374.

6 Seggel, K. A., *Folia Haemat.*, 1934, **52**, 250.

7 Seggel, K. A., *Klin. Wchnschr.*, 1936, **15**, 574.

8 Seggel, K. A., *Klin. Wchnschr.*, 1936, **15**, 296.

9 Müller-Neff, H., *Folia Haemat.*, 1936-37, **56**, 18.

10 Watson, C. J., and Clarke, W. O., PROC. SOC. EXP. BIOL. AND MED., 1927, **25**, 65.

11 Watson, C. J., The Pyrrol Pigments, with Particular Reference to Normal and Pathologic Hemoglobin Metabolism. Hal Downey, *Handbook of Hematology*, Paul B. Hoeber, New York, 1938, 4, 2445.

12 Langen, C. D. de, and Grotepass, W., *Acta Med. Scand.*, 1938, **97**, 29.

contents of the tube were thoroughly mixed by shaking. The mixture was allowed to stand overnight and then centrifuged for 15 min. The clear, dark colored supernatant fluid was decanted into a second 15 cc centrifuge tube and 5 cc of 5% HCl added with thorough mixing. This mixture was centrifuged 5 min to separate the two layers. The upper layer of ethyl acetate was floated off carefully by addition of distilled water. The 5% HCl solution was then placed before a source of ultraviolet light (3200-4000 A) and the protoporphyrin estimated as 0, ±, +, 2+, 3+ or 4+ by visual comparison of the fluorescence of the solution with standard protoporphyrin solutions.

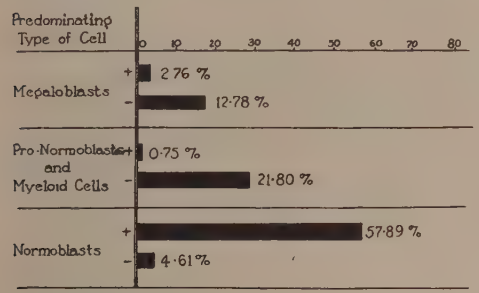

FIG. 1.

The highest incidence of protoporphyrin was coincidental with the preponderance of immature normoblastic red cells in 133 sternal marrow specimens.

Observations. The different marrow samples showed variations in cellularities and in the amount of protoporphyrin. There was no direct relationship between the total number of nucleated elements and the amount of protoporphyrin, but it appeared to be related to the presence of certain types of cells. As Fig. 1 shows, protoporphyrin occurred most consistently in marrow samples which consisted predominantly of immature normoblastic red cells. The included data were derived from the examination of 133 sternal bone marrow specimens, 96 of which were obtained from 12 cases of pernicious anemia before and during treatment with liver extract. Twenty-six sternal marrow samples were obtained from patients with secondary type of iron deficiency anemias showing normoblastic stimulation. Twenty-three bone marrow samples revealed the presence of protoporphyrin and in 3 instances no detectable amount was present. One hyperplastic normoblastic bone marrow of a case of sickle cell anemia, and one case of phosphorus poisoning with jaundice were strongly positive. Two bone marrows of sprue with a preponderance of pronormoblastic cells failed to show the presence of protoporphyrin. In 2 marrows from cases of aplastic anemia, there was only a small amount of detectable protoporphyrin present in one. From 5 cases of leucemias with hyperplastic myeloid or lymphoid marrow protoporphyrin could not be demonstrated in 4 instances.

The bone marrow of 12 patients with pernicious anemia was examined, showing a preponderance of megaloblastic cells. These specimens were negative for protoporphyrin with a few exceptions, although some of them were examined repeatedly. Serial sternal marrow samples were taken at 24-hr intervals after the beginning of the liver treatment. One typical chart (Fig. 2) illustrates that within 24-48 hr after the injection of 60 U.S.P. units of liver extract (Lederle) there were marked fluctuations in the number of nucleated marrow cells, the majority of which were of the normoblastic series.

Before liver extract was administered the megaloblastic cells were predominate in the marrow and characterized by finely dis-

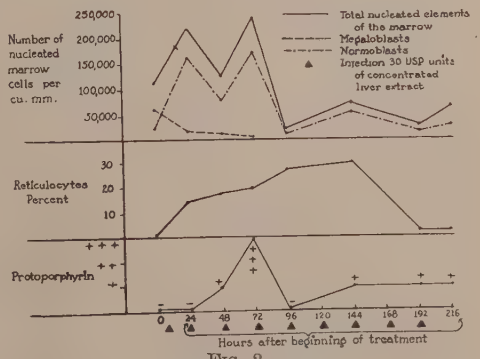

FIG. 2.

Illustrates the serial bone marrow studies in a case of pernicious anemia before and at frequent intervals after the first liver injection. The chart shows that the appearance of protoporphyrin was observed simultaneously with the proliferation of immature normoblastic cells.

tributed chromatin in the nucleus and blue cytoplasm. Twenty-four to 48 hr later these cells were replaced by numerous normoblasts with a distinctly coarse chromatin nuclear pattern and blue cytoplasm. About the same time there was an increase in the amount of protoporphyrin. This was followed by a rapid rise in the number of reticulocytes in the marrow.

It should be emphasized, that a simple comparative method which was used in this work for the protoporphyrin determinations provided only the roughest kind of quantitative data as to the relative amount of protoporphyrin of the different marrow samples. After these observations, however, more accurate quantitative determinations are in progress with the use of the Coleman Photofluoro-meter.

Comment. The present observation that the immature red blood cells of normoblastic origin in the bone marrow exhibit an increased amount of protoporphyrin is well in accord with the other observations that an increased amount of protoporphyrin was directly correlated with the number of reticulocytes,[10] or young erythrocytes.[9] It is noteworthy that contrary to the findings of others,[3] we found little or no protoporphyrin in the megaloblastic marrow.

It has been noted that patients with pernicious anemia in relapse had a low protoporphyrin content of the blood and that the values became elevated during the early stages of liver induced remission.[13–15] Increased amounts of protoporphyrin in the urine and feces of patients with pernicious anemia has also been reported.[13, 18–20]

By perfusion of surviving livers with protoporphyrin solution it was shown that a varying fraction of the protoporphyrin was converted into coproporphyrin.[21] This was not confirmed by other workers and it was shown that the porphyrin formed under these circumstances is not coproporphyrin, but one of the pseudodeutero-porphyrins.[22,23] Nevertheless, coproporphyrin I has been found to be excreted normally and in increased amounts in the urine and feces obtained from pathological cases showing increased hematopoietic activity.[16,17,19,24,25] It is unlikely that the coproporphyrin I is a hemoglobin derivative, since all the respiratory pigments are Type III compounds. Therefore, it was assumed in accordance with the theory of the dualism of the porphyrins that there is a simultaneous construction of Type I and Type III porphyrins in direct proportion. The coproporphyrin I excretion was regarded as an index of blood regeneration.[25,26]

It is likely that protoporphyrin accumulates at the site of hemoglobin formation as an intermediate substance. It has also been suggested[7,12,14] that the increased amount of protoporphyrin is simply an expression of mild iron deficiency.

In the present study we found an increased amount of protoporphyrin concomitant with a large number of newly formed normoblastic cells. The relationship between the amount of protoporphyrin and the number of reticulocytes, however, was not clear. In some instances the increase of protoporphyrin preceded the rise in the number of reticulocytes. It is possible that during the marked normoblastic proliferation there are red cells containing improperly formed respiratory pigment which, even though they were not

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14 Vannotti, A., *Porphyrine und Porphyrinkrankheiten*, J. Springer, Berlin, 1937.

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16 Duesberg, R., *Arch. f. exp. Path. u. Pharmakol.*, 1931, **162**, 249.

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18 Watson, C. J., *J. Clin. Invest.*, 1937, **16**, 383.

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21 Van den Bergh, H. A. A., Grotepass, W., and Revers, F. E., *Klin. Wchnschr.*, 1932, **11**, 1534.

22 Watson, C. J., Pass, I. J., and Schwartz, S., *J. Biol. Chem.*, 1941, **139**, 538.

23 Salzburg, P., and Watson, C. J., *J. Biol. Chem.*, 1941, **139**, 593.

24 Watson, C. J., *J. Clin. Invest.*, 1935, **14**, 116.

25 Dobriner, K., and Rhoads, C. P., *J. Clin. Invest.*, 1938, **17**, 95.

26 Dobriner, K., and Rhoads, C. P., *J. Clin. Invest.*, 1938, **17**, 105.

reticulocytes, might contain protoporphyrin. In this connection it is of interest that the amount of non-respiratory iron porphyrin compounds was increased in immature red cells.[27] Furthermore, during liver-induced remission in patients with pernicious anemia, there has been observed a precipitous fall in serum iron values[28] which preceded, but was almost reciprocal with, the rise in reticulocytes.[29]

Summary. The occurrence of protoporphyrin was studied in 133 sternal bone marrow specimens. Protoporphyrin was most regularly present in marrow samples containing predominately normoblastic young red blood cells.

Ninety-six sternal bone marrow specimens from 12 patients with Addisonian pernicious anemia before and after liver extract injection were obtained by serial sternal punctures at 24-hr intervals. The appearance of protoporphyrin was coincidental with the increase of immature red cells of normoblastic type in the marrow.

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28 Heilmeyer, L., and Plötner, K., *Serumeisen und die Eisenmangelkrankheit*, G. Fischer, Jena, 1937.

29 Moore, C. V., Doan, C. A., and Arrowsmith, W. R., *J. Clin. Invest.*, 1937, **16**, 627.

13967

Blood Iodine in Dogs Receiving Thyroxin or Phlorhizin.

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Since an increase in the basal metabolic rate may be due to various causes, supplementary tests have long been sought which would aid in differentiating thyroid disease from other conditions in which a rise in metabolism occurs. The intimate connection between iodine metabolism and the thyroid suggests that blood iodine determinations might be of particular value for this purpose. Extensive use of microdeterminations of iodine in balance studies and blood analyses has enabled several investigators to extend our knowledge of iodine metabolism and of the course of thyroid disease.[1,2] The same studies also indicate some of the limitations to be expected of blood iodine determinations. Known cases of hyperthyroidism, following a period of mobilization and loss of thyroid iodine, may show normal blood iodine levels but retain their high metabolic rate. Thus there appears to be no immediate relationship between the concentration of iodine in the blood and the basal metabolic rate at a given time. Occurrence of high values for blood iodine in non-thyroid conditions has also been mentioned.[2]

It therefore appeared of interest to study the relationship between basal metabolic rate and blood iodine in dogs receiving injections of thyroxin or phlorhizin. The calorigenic response to thyroxin is presumably due to the injected hormone itself. Phlorhizin, on the other hand, although it produces its principal effect in both normal and thyroidectomized dogs, sharply increases the metabolic rate only if the thyroid is present.[3,4] Can involvement of the thyroid in this experimental condition be detected by determining blood iodine?

In the following experiments normal adult

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female dogs, thoroughly accustomed to living in metabolism cages were used. The first group (Table I) received a stock diet of cracker meal, casein, corn oil, yeast, bone ash, and Karr's salt mixture[5] without added

TABLE I.
Blood Iodine of Dogs After Intravenous Injections of 10 mg of Thyroxin.

	Blood iodine mcg/100 cc			
Hr after thyroxin	dog 11 16.0 kg	dog 11	dog 32 12.8 kg	dog 35 13.9 kg
Control	1.0	3.1	2.1	2.1
"	2.1	2.1	2.1	1.1
0.5	159.		93.	200.
1	136.		88.	169.
2	134.	76.	73.	164.
4	99.	70.	56.	108.
8	59.		39.	86.
12	50.	40.	29.	64.
24	29.	29.6	19.1	40.
48		9.5	5.3	15.8
72	2.1	6.3	7.4	7.4
96		7.4	3.1	5.3
120		5.3	2.1	5.3
144		4.2		2.1
168		4.2		
192		3.1		

iodine. Crystalline thyroxin (Squibb) was carefully weighed in a 4 cc test tube, dissolved therein by adding a drop of 10% sodium hydroxide and water, transferred with rinsings to a 10 cc syringe, and injected into a leg vein. Blood samples were drawn from the jugular veins. Determinations of iodine were made by a method similar to that of Trevorrow and Fashena,[6] but after the initial oxidation the digest was transferred to a one-piece all glass outfit for distillation without aeration. The final reoxidation to iodate and titration of this were performed as described elsewhere.[7]

The rapidity with which amino acids disappear from the circulation when protein digests are injected intravenously is well known. In keeping with this the blood iodine during the first 3 min after injections of thyroxin never exceeded 302 mcg per 100 cc. Had the 10 mg of injected thyroxin been distributed uniformly through the circulation and remained there, the value should have exceeded 600 mcg per 100 cc of blood in these animals. It is however equally worthy of note that while half of the thyroxin was never detected and 90% of the remainder disappeared from the circulation in 24 hr the presence of an increased amount of iodine was detectable in the blood for 3 to 6 days.

In 2 highly trained animals the calorigenic action of 10 mg doses of thyroxin was determined, by using a mask[8] and the Tissot method. We are indebted to Miss Annette Moore for the numerous gas analyses. In 7 experiments the time curve of the calorigenic effect was essentially that previously reported by others.[9] It may therefore be stated that during the first 24 hr following intravenous injection of 10 mg of thyroxin the metabolic rate is rising rapidly while blood iodine is falling rapidly; during the second and third 24-hr periods the metabolic rate remains high, while blood iodine continues a rapid return to normal. There is thus no parallelism between blood iodine and the metabolic rate in such experiments.

TABLE II.
Blood Iodine of Dogs Before and During Phlorhizin Injections.

	Blood iodine, mcg per 100 cc		
	Dog 14	Dog 11	Dog 35
Before phlorhizin			
5 days			3.2
4 "	1.5		4.2
3 "	(4.2)		4.2
2 "	1.5	1.1	5.3
1 "	2.1	2.1	3.5
During phlorhizin			
1 day	2.1	1.1	1.1
2 days	1.5	1.1	5.3
3 "	2.1	0.9	1.1
4 "	2.1	1.5	3.2

Results obtained in phlorhizinized dogs are shown in Table II. These animals received 750 g of horse meat and 40 g of bone ash daily, so that the diet would be comparable with the meat diets used in earlier studies

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[6] Trevorrow, V., and Fashena, G. J., *J. Biol. Chem.*, 1935, **110**, 29; 1936, **114**, 351.

[7] Gaebler, O. H., and Baty, M., *Ind. and Eng. Chem., Anal. Ed.*, 1941, **13**, 442.

[8] Gaebler, O. H., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 500.

[9] Wilhelmj, C. M., and Boothby, W. M., *Am. J. Physiol.*, 1930, **92**, 568.

on the effects of phlorhizin on the respiratory metabolism of dogs. Only 10 cc samples of blood were used, and since the blood iodine values are all at the normal level the titrations were too small for a high degree of accuracy. However, additions to blood of thyroxin equivalent to 10 mcg of iodine per 100 cc were recovered with an accuracy of over 90%. It is evident that no definite rise in blood iodine occurred in the phlorhizinized animals. This is especially significant if one considers the fact that the basal metabolic rate of intact dogs receiving this amount of phlorhizin increases 70%.[3,4] In cases of thyroid disease presenting such an elevation of metabolism the blood iodine may be increased 20 times or more.

Conclusion. Neither in normal dogs receiving 10 mg doses of thyroxin intravenously, nor in intact dogs receiving phlorhizin, is there any parallelism between the blood iodine level and the basal metabolic rate. The former result conforms with expectations, since the blood is not the site of action of thyroxin. The latter finding is more unexpected, since the increase in metabolism which phlorhizin produces in dogs is very large and is abolished by thyroidectomy.

13968

Effect of Direct Applications of Tyrothricin and Allantoin to Cells *in vitro*.*

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Recent interest in the direct application of sulfonamide crystals to wound surfaces and to body cavities has stimulated research on the effect of such treatment upon cells cultivated *in vitro*.[1,2] In spite of the higher resistance of embryonic cells in tissue cultures to the action of inhibitory agents, workers in this field hold that *in vitro* experiments offer stringent and easily controlled conditions for the analysis of drug toxicity.

In addition to the sulfonamides, a new class of compounds characterized by high bactericidal action is being derived from microörganisms. Of these, tyrothricin and its derivatives, are described as unsafe at present for general use, especially when brought in direct contact with the blood stream.[3,4] Tyrothricin, however, has been found not to interfere with the healing of wounds[3] and therefore may become useful in this direction.

Somewhat better known, allantoin, the chemical associated with the benefits of maggot therapy, has already proved useful at 2% concentrations in stimulating the granulation of chronic suppurative wounds.[5,6] Its action does not appear to be bactericidal and therefore it has been used in association with some disinfectant. Veal[7] has found that allantoin combined with sulfanilamide in a greaseless base was extremely valuable

*The author wishes to express his appreciation to Miss M. Capouya and to Miss F. I. Greenleaf for technical assistance. Thanks are also due for the gift of Tyrothricin by Merck and Co., and of Allantoin by the Abbott Laboratories.

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6 Greenbaum, F. R., *Am. J. Surg.*, 1936, **34**, 259.

7 Veal, Ross, *Med. Ann. of Dist. of Col.*, 1941, **10**, 2.

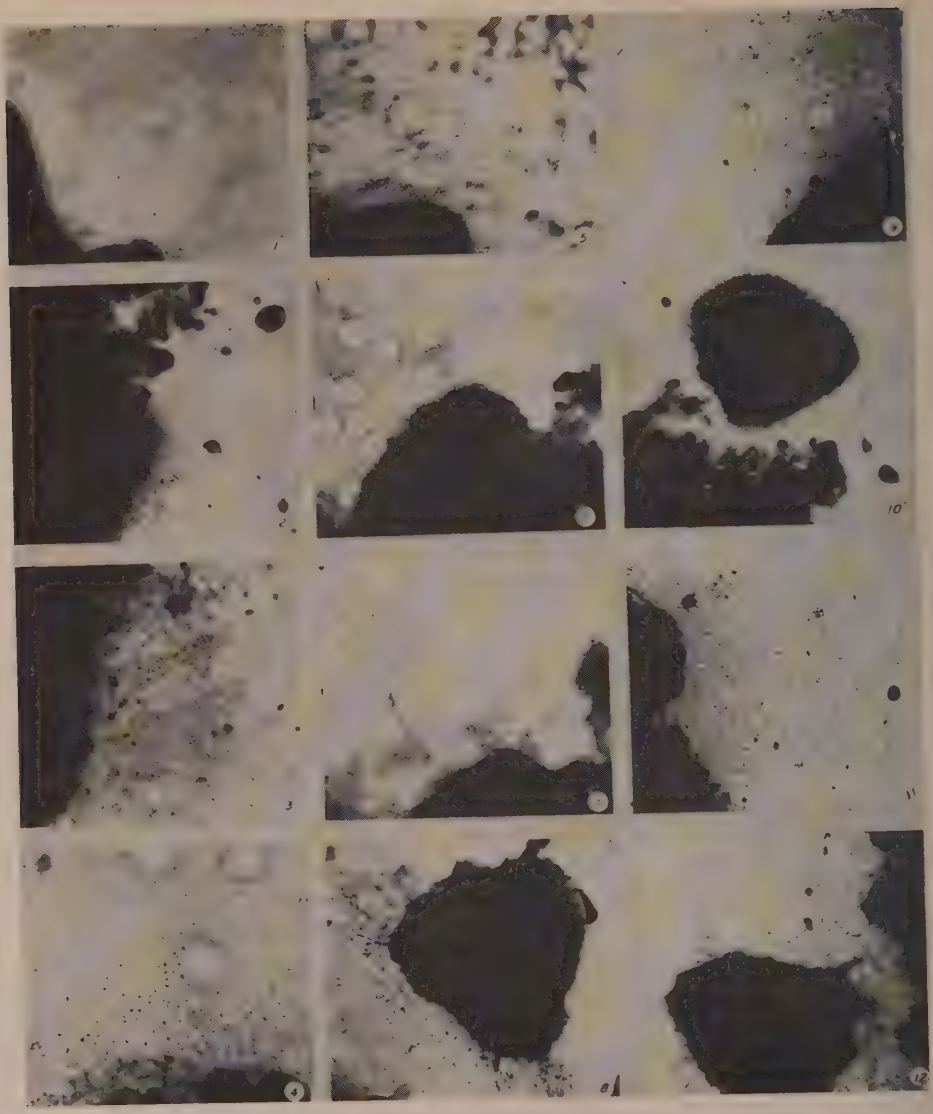

PLATE 1.

Explanation of Figures.

FIG. 1. *Tyrothricin.* Active migration of fibroblasts through a field of powder (left side) 72 hr after direct application to a freshly cut heart fragment. ×30.

FIG. 2. *Tyrothricin.* Growth of fibroblasts in a heart culture after 24 hr incubation. Photograph taken immediately after application of the powder which appears as dense round masses. ×30.

FIG. 3. *Tyrothricin.* Same culture as in Fig. 2 but photographed 48 hr later. ×30.

FIG. 4. *Tyrothricin.* Active migration of leucocytes from a spleen fragment (lower left) treated with powder (lower right) after 48 hr incubation. ×30.

FIG. 5. *Allantoin.* Active growth of fibroblasts in a field of crystals 72 hr after application to a freshly cut heart explant. ×30.

FIG. 6. *Allantoin.* Growth of fibroblasts in a heart culture after 24 hr incubation. Photograph taken immediately after application of crystals (right side). ×30.

FIG. 7. *Allantoin.* Same culture as in Fig. 6 but 48 hr later. Migration of fibroblasts is seen to have continued following treatment. ×30.

FIG. 8. *Allantoin.* Migration of leucocytes from a spleen fragment in the presence of crystals. 24-hr culture. ×30.

FIG. 9. *Tyrothricin-Allantoin.* Active growth of fibroblasts 72 hr after treatment of a freshly prepared explant of chick heart. ×30.

FIG. 10. *Tyrothricin-Allantoin.* Growth of fibroblasts in a heart culture after 24 hr incubation. Photograph taken immediately after treatment with dry mixture of tyrothricin and allantoin (lower left area). ×30.

FIG. 11. *Tyrothricin-Allantoin.* Same culture as in Fig. 10 but 48 hr later showing continued active growth of fibroblasts. ×30.

FIG. 12. *Tyrothricin-Allantoin.* Migration of leucocytes from a spleen fragment in the presence of powder mixture (right) after 24 hr incubation. ×30.

TABLE I.

Effect of Tyrothricin and Allantoin Powder Applied Individually or in Combination to Fibroblasts and Leucocytes Grown Cultivated *in vitro*.

Compound used	Tissue	Individual results						
Tyrothricin	Heart fresh explant	+++	++	+++	++++			
	" 24-hr growth	++++	++++	++++	++	+	++	
		++++	++++	++++	++	+	++	
	Subcultured		+++		+++		+++	
	Spleen	+++		++	++++	+++	++	
		+++		++	++++	+++	++	
Allantoin	Heart fresh explant	++++	++++	+	+++			
		++++	++++	+	+++			
	" 24-hr growth	+++	+++					
	Spleen	++	++	++	++	+++	++	
Equal parts tyrothricin and allantoin	Heart fresh explant	++++	+++	+++	++++			
	" 24-hr growth	++++	+++	++++	++++			
	Spleen	++	++	+	+++	+++	+++	

in the preparation of extensive burns for skin grafts.

It was the object of the present study to submit tyrothricin, allantoin and a combination of equal parts of both compounds to the test of direct application to cells grown in tissue culture.

Experimental. The technic employed was identical with that described by Pomerat, Capouya and Greenleaf[2] for a similar study of sulfonamide compounds. Hanging-drop preparations were made using equal parts of rooster plasma and a 20% extract in Tyrode's solution, of chick embryos incubated for 7 to 10 days. Three types of cultures used were: (1) freshly explanted fragments from the ventricle of chick embryos incubated for 8-10 days, (2) heart explants prepared as in (1) but grown 24 hr before treatment, and (3) spleen fragments of 1-2 day old chick from which leucocytic migration could be observed. The compounds to be tested were placed on one side of the explant in a quantity sufficient to exceed saturation at 38°C for the entire period of observation. One untreated control culture was set up for each group of 5 experimental preparations.

Photomicrographs were taken at the time of treatment and at various intervals up to 72 hr for the measurement of fibroblast growth and to 24 hr for the migration of leucocytes. The object of the study was to test growth *versus* non-growth in the presence of the undissolved compounds. No exact quantitative measurement was made of the rate of growth but this was roughly estimated in terms of control cultures and is reported by means of plus signs, the largest rate being given as ++++.

Results. Table I and Plate 1 illustrate the results obtained for the 3 types of tissue preparations in relation to the compounds studied.

(A) *Tyrothricin.* When this is added to the culture medium it tends to spread moderately and to form a film with a very thin edge. Cells growing on the surface of a clot which is covered by a very thick layer of

powder appear to be mechanically obstructed and show almost complete inhibition. Fibroblasts and leucocytes which are in the immediate vicinity, but which are not heavily covered, are not damaged and undergo proliferation typical of control cultures. This was found true for freshly cut fragments of chick ventricle and spleen, as well as for heart cultures which had proved a capacity for active growth following 24-hr incubation.

(B) *Allantoin.* Heart tissue dusted with allantoin immediately after fragments were set in clotted medium or after fibroblasts had been growing for 24 hr were not inhibited, but growth did not progress beyond that of the controls. Allantoin did not prevent chance contamination of some cultures. These, however, are not included in the table since the data were restricted to cultures which remained sterile throughout the experiment.

The migration of leucocytes from spleen fragments was not as active in allantoin treated cultures as in the controls, but complete inhibition was not observed in any of the cultures so treated which were studied.

(C) *Mixture of tyrothricin and allantoin in equal proportions.* While the total number of test cultures is small and the method of evaluating the amount of growth is not highly quantitative, some impressions are justified. Fibroblasts appeared to grow somewhat more actively than in either of the components of the mixture when used alone. This growth, however, did not exceed perceptibly that of the controls, so that cell stimulation is not a justifiable interpretation. Leucocyte migration from spleen was as active as in the presence of allantoin alone, but it was somewhat less active than in the presence of tyrothricin alone. Leucocytic activity, however, was far less than that of control cultures.

Discussion. In spite of its well known hemolytic effect,[3] tyrothricin does not appear to inhibit materially either mitosis or migration of fibroblasts, or the activities of leucocytes following its direct applications to tissue culture media.

Three cultures treated with tyrothricin powder after growth for 24 hr and then incubated for 48 hr were subcultured and exhibited growth in the second passage equal to that obtained in controls.

Heart fibroblasts treated with allantoin have been studied in tissue cultures by Shipp and Hetherington.[8] These authors report the addition of allantoin to Tyrode's solution before the extraction of the embryos in quantities sufficient for a final dilution of 0.5%. This method differs from that of the present study wherein allantoin crystals were dusted directly onto the clotted culture medium. The author's methods were not sufficiently quantitative to corroborate Shipp and Hetherington's statement that allantoin is slightly stimulating to fibroblasts grown *in vitro,* but they do indicate that crystals of this compound are relatively non-damaging to cells in tissue cultures.

These results resemble those obtained for similar experiments using sulfonamide compounds[1,2] in which the less soluble members of the group exhibited extraordinarily low toxicity when dusted directly onto cells cultured *in vitro.*

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13969 P

Gallstone Formation and Intake of B Vitamins in Cholesterol-Fed Guinea Pig.

RUTH OKEY.
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Thiamin, riboflavin, pantothenic acid and biotin are all reported to produce fatty livers in the rat; choline, inositol, and pyridoxine to prevent them.[1] Biotin fatty livers alone have a high cholesterol ester content.[2]

The cholesterol-fed guinea pig develops a fatty liver, enlarged spleen and a severe anemia. Liver damage can to some extent be judged by red cell count.[3]

Vitamin requirements of the guinea pig are not entirely known. Findings reported here are the indirect results of an attempt to improve the nutrition of our animals and so make it possible to study the lesions remaining after a period of cholesterol feeding.

Diet "A" had been planned to parallel in respect to protein and fat content an egg yolk diet previously used. It supported growth in control animals slightly less well than our stock diet. Possible shortcomings seem to be low riboflavin and pantothenic acid and too much fat.

Diet "B" in which the amounts of these components were adjusted as indicated below, increased apparent well being in both control and cholesterol-fed animals. We were greatly surprised, therefore, when the cholesterol-fed animals on this diet showed

TABLE I.
Basal Diets Fed *ad libitum*.

	Group "A"		"B," "C," "D"	
	Control	Cholesterol	Control	Cholesterol
Casein, commercial, parts	20	20	20	20
Fat ("Crisco") "	15	15	12.5	12.5
Wheat bran "	5	5	5	5
Dried brewers yeast "	10	10	10	10
Hubbels salt "	2.5	2.5	2.5	2.5
Agar "	2.5	2.5	2.5	2.5
Cornstarch "	45	44	47.5	46.5
Cholesterol "	0	1.0	0	1.0

10 g of these diets, or a minimum daily intake, assayed by microbiological technic:* 90 γ thiamin, 90 to 120 γ riboflavin, 500 γ nicotinic acid and 140 γ pantothenic acid.

TABLE II.
Daily Doses of Vitamin Supplements, Given by Pipette.

Group A	¼ g "cerophyll"* ≅ 2.5 γ pantothenate; 2.5 γ thiamin; 6 γ riboflavin; grass juice factor, "K," etc. 25 γ thiamin 3 cc orange juice + 2.5 mg ascorbic acid 75 I.U. "A" as carotene 25 I.U. "D" as irradiated ergosterol 1 drop wheat germ oil
Group B	As group "A" plus 25 γ riboflavin and 125 γ pantothenate
Group C	As group "A" plus 125 γ pantothenate only
Group D	As group "A" plus 25 γ riboflavin only

*Microbiological assays by Dr. Relda Cailleau of this laboratory.

* Assistance of the Work Projects Administration is gratefully acknowledged. (Project 50-12417-A-24.)

1 Engel, R. W., *J. Nutr.*, 1942, **24**, 175.

2 McHenry, E. W., and Gavin, G., *Fed. Proc.*, 1942, **7**, 124.

3 Okey, R., and Greaves, V. D., *J. Biol. Chem.*, 1939, **111**, 729.

TABLE III.

Summary of data	Cholesterol fed animals only			
Groups	A	B	C	D
No. of pigs	11	12	4†	5
No. of animals with gallstones	0	10*	1	5

*No satisfactory autopsy on 2 remaining animals which died at night. Gall bladders burst and contents were lost.

†Bile murky but no hard stones in 3 animals.

an abrupt fall in red cell count without the usual preliminary loss in weight and other evidences of illness, and when the autopsies showed gallstones.

Guinea pig bile contains relatively little lipid. These gallstones were rich in calcium phosphate, although they contained some cholesterol. Irritation of the membranes of the gall bladder and the biliary passages was common and some impaction of ducts was observed in cholesterol-fed animals. No stones were found in control animals.

If this high incidence of gallstones is to be attributed to the relatively small increase in riboflavin intake, these data suggest that a critical level of tolerance was exceeded. That this is not a definite figure but may vary with the intake of other dietary factors is also suggested.

Examination of autopsy notes for guinea pigs of our previous series revealed that all the animals which had gallstones were on diets which probably contained extra riboflavin. Incidence was especially high when unextracted casein and/or egg yolk protein had been fed at levels exceeding 25%. We thought at the time that the gallstones might be due to high protein intake.

The whole problem of the relationship of the intake of B vitamins to tolerance for foods high in cholesterol obviously needs further investigation. Preliminary publication of our findings seems desirable at this time because of the wide and indiscriminate use of very high dosages of preparations of the various B factors.

13970 P

Mortality in Severe Experimental Burns as Affected by Environmental Temperature.

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Our armed forces are on duty in all parts of the globe, and are subjected to all extremes of temperature—from arctic to equatorial. Burns from bomb flashes, gasoline fires and escaping steam are common in modern war, yet we have been unable to find any observations on the effect of environmental temperature on the early mortality resulting from such events. The only comment on the environmental management of the burned patient is that he should be kept warm because of the fall in body temperature commonly accompanying shock.[1] While engaged in a study of certain pathological and chemical changes following experimental burns in dogs, the results of which will be reported elsewhere, we noted an increased mortality during several hot days during the summer months when the temperature rose to 95°F. These observations seemed to indicate that environmental temperature actually did affect mortality.

As an experimental approach to this problem, we have burned rats under controlled conditions, placed them at 4 different temperatures, and determined their survival after 24, 48 and 72 hr. Our major interest was in the mortality during the first 24 hr, since

secondary factors undoubtedly affect survival after this initial period. The striking results obtained prompts this preliminary report.

Adult rats were anesthetized with nembutal intraperitoneally (.033 g per 100 g of body weight) and the lower portion of their body, to the axilla, immersed in hot water. They were held vertically by two forceps—one at the scruff of the neck, and the other grasping the proximal end of the tail. Immersion lasted 7 sec. Two degrees of burns were studied: (1) 152 rats in water at 80°C (176°F), and (2) 78 rats in water at 90°C (194°F). Immediately after immersion the rats were kept in rooms held at one of four temperatures, 32°, 55°, 75° and 99°F. These room temperatures showed a maximum variation of plus or minus 2°F. The mortality after 24, 48 and 72 hr was observed.

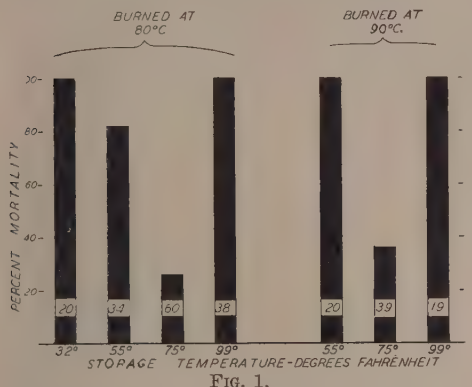

FIG. 1.

Twenty-four hour mortality of burned rats. The figures on the columns indicate the number of rats used in determining the mortality figure.

Results are apparent from the accompanying chart. All animals placed in rooms at 32° and 99°F were dead within 24 hr. At 55°F, 100% of those burned at 90°, and 82% of those burned at 80°C were dead within 24 hr. The 18% in the latter group which survived the 24-hr period were still alive at the end of 72 hr. Of those placed in a room at 75°F only 26% died in the first 24 hr among the 80° burns, 65% were dead at the end of 48 hr and 83% in 72 hr. Of those burned at 90° and held at 75°F, the mortality was 36%, 95% and 100% at 24, 48 and 72 hr respectively.

These observations indicate the importance of environmental temperature in the early mortality following extensive body burns. Apparently the most favorable temperature is about 75°F, which is actually a little higher than the level assumed to be the normal average for human comfort. It is obvious that the commonly used heat tent which increases the environmental temperature above this point will be deleterious by increasing mortality in severe burns. On the other hand, these findings suggest the importance of lowering room temperature of burned patients in climatic extremes, when the environmental temperature rises much above this point.

Summary. Environmental temperature has a decisive influence on the 24-hr mortality following a severe cutaneous burn. The lowest mortality (32%) occurred at 75°F and increased to 100% with either an increase or decrease of 20°F.

13971 P

A Cancerogenic Extract from Human Bile and Gall Bladders.*

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The possibility that human bile might contain substances which have cancerogenic activity was suggested by the discovery that a powerful cancerogen, methylcholanthrene, could be made from bile acids,[1-3] by the incidence of carcinoma of the biliary system, where comparatively few cells give rise to numerous tumors, and by discovery that extracts of human liver have cancerogenic activity.[4] Some of this material might be excreted in the bile and induce tumors in the biliary passages, and in the gastrointestinal tract.

Human bile has not previously been reported to induce sarcomas at the site of injection. Using ox bile, Turner[5] induced 1 sarcoma in 75 mice that lived over 34 weeks. Bürger and Uiker[6] induced only leukemia-like changes in the liver and spleen of mice with an emulsion of human bile. Neufach and Shabad[7] also induced no tumors at the site of injection of a benzene extract of human bile, but stated that the incidence of various tumors in distant organs was greater than in controls.

Methods. Human gall bladder bile and some of the gall bladders from which it was obtained were collected from adults. Approximately 40% of these persons died with malignant tumors. The dried residue, weighing 2400 g, was saponified with alcoholic potassium hydroxide by refluxing on a steam bath for 24 hr. After repeated extractions with ethylene dichloride the extracts were combined and dried over anhydrous sodium sulfate. The extract was then filtered and the ethylene dichloride distilled in partial vacuum. The residue was resaponified with alcoholic potassium hydroxide for 4 hr, and then extracted, dehydrated, filtered, and distilled as before. The nonsaponifiable residue weighed 38.5 g.

This extract was tested for cancerogenic activity by subcutaneous injection. Fifty-three mice were each injected with 250 mg of the extract in 0.75 cc of sesame oil. The injections were repeated at 6 weeks. This sesame oil has not induced tumors in this stock of mice when injected alone or with various tissue extracts prepared by this method.[4,8,9] The mice were from 107 to 127 days old. Something about their susceptibility to spontaneous and induced tumors has been given in several reports.[4,9,10] They do not have spontaneous sarcomas, other than lymphosarcomas.

Results. The mice lived to the periods of

TABLE I.
Cancerogenicity of a Bile Extract.

Time in mo.	Living mice ♂	Living mice ♀	Living mice Total	Deaths with sarcoma
0	13	40	53	
6	6	39	45	
12	1	34	35	
15	0	30	30	1
18	0	25	25	
21	0	13	13	1
24	0	7	7	3
27	0	3	3	
29	0	2	2	

* This work was aided by grants from the National Advisory Cancer Council and from the Commonwealth Fund.

1 Cook, J. W., and Haslewood, G. A. D., *J. Chem. Soc.*, 1934, p. 428.

2 Wieland, H., and Dane, E., *Ztsch. f. physiol. Chem.*, 1933, **219**, 240.

3 Fieser, L. F., and Newman, M. S., *J. Am. Chem. Soc.*, 1935, **57**, 961.

4 See review: Steiner, Paul E., *Cancer Research*, 1942, **2**, 425.

5 Turner, F. C., *U. S. Public Health Reports*, 1939, **54**, 1603.

6 Bürger, M., and Uiker, R., *Klin. Wchnschr.*, 1937, **16**, 334.

7 Neufach, S. A., and Shabad, L. M., *Bulletin de Biologie et de Médecine Experimentale de L'URSS*, 1938, **6**, 259.

8 Steiner, Paul, E., *Cancer Research*, 1942, **2**, 181.

9 Steiner, Paul E., *Cancer Research*, in press.

10 Steele, R., Koch, F. C., and Steiner, P. E., *Cancer Research*, 1941, **1**, 614.

time shown in the table. Five sarcomas occurred at the site of injection of the extract in mice dying in the 14th, 20th, 23rd, 23rd, and 24th months after the first injection. Two mice are alive and without visible tumors at 29 months. The tumors were spindle or spindle and mixed cell sarcomas. They resemble sarcomas induced by cancerogenic chemicals in the subcutaneous tissues in other experiments, both in their morphology and growth.

Comment. The occurrence of 5 sarcomas in 32 mice (percentage yield of 15.6) indicates, despite the long induction time, that this extract had a fair degree of cancerogenic potency. Each mouse was injected with the non-saponifiable lipid residue from a total of 31.05 g of dried gall bladder bile and dried gall bladders themselves. This represents a large volume of gall bladder bile and an even larger amount of unconcentrated bile. Other extracts of bile, prepared by different methods, either have not induced tumors or are still under test. They will be described in a later report.

Although Cook, Kennaway, and Kennaway[11] reported the induction of tumors with desoxycholic acid, Shear *et al.*[12] and others have failed to induce tumors with bile acids. It is highly improbable that the tumors reported here were induced by bile acids because the extract was made with a lipid solvent from a strongly alkaline aqueous-alcohol solution in which the bile acids should remain as salts.

[11] Cook, J. W., Kennaway, E. L., and Kennaway, N. M., *Nature*, 1940, **145**, 627.

[12] Shear, M. J., Leiter, J., and Perrault, A., *J. Nat. Canc. Inst.*, 1941, **2**, 99.

13972

"Folic Acid" in Nutritional Achromotrichia.

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The concept of synthesis of vitamins by the bacterial flora of the intestine seems to have originated in work on the phenomenon of refection.[1] Nutritional investigation of the problem received a great impetus with the announcement[2] that sulfaguanidine, an antibacterial agent which is poorly absorbed, reduced the intestinal flora.

Black *et al.*[3] demonstrated reduced growth rate in rats on purified diets containing synthetic vitamin mixtures and sulfaguanidine. Various other investigators confirmed and extended these observations.[4-9] The nature of the deficiencies produced by the inclusion of sulfonamides in synthetic diets has recently been disclosed. Black *et al.*[10] demonstrated effects by both vitamin K and p-aminobenzoic acid, the vitamin K correcting a hypoprothrombinemia and the para-aminobenzoic acid improving growth. Biotin deficiency in rats fed sulfonamide-containing

[1] Guerrant, N. B., Dutcher, R. A., and Tomey, L. F., *J. Biol. Chem.*, 1935, **110**, 233.

[2] Marshall, E. K., Jr., Bratton, A. Calvin, White, H. J., and Litchfield, J. T., Jr., *Bull. Johns Hopkins Hosp.*, 1940, **67**, 163.

[3] Black, S., McKibbin, J. M., and Elvehjem, C. A., Proc. Soc. Exp. Biol. and Med., 1941, **47**, 308.

[4] Mackenzie, J. B., Mackenzie, C. G., and McCollum, E. V., *Science*, 1942, **94**, 518.

[5] Dann, W. J., *J. Biol. Chem.*, 1941, **141**, 803.

[6] Daft, F. S., Ashburn, L. L., Spicer, S. S., and Sebrell, W. H., *U. S. Public Health Rep.*, 1942, **57**, 217.

[7] Welch, A. D., *Fed. Proc.*, 1942, **1**, 171.

[8] Light, R. F., Cracas, L. J., Olcott, C. T., and Frey, C. N., *J. Nutr.*, 1942, Nov., 24.

[9] Martin, G. J., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 56.

[10] Black, S., Overman, R. S., Elvehjem, C. A., and Link, K. P., *J. Biol. Chem.*, 1942, **145**, 137.

diets was reported by Daft *et al.*[11] This fact was confirmed by Nielson and Elvehjem[12] who found in addition a powerful growth stimulating action of "folic acid."

Black rats, 40 to 50 g in weight, were placed on diets having the basic composition: vitamin free casein, 18; sucrose, 67; salts, 4.0; butter fat, 9.0; cod liver oil, 2. The basic vitamin supplements were added at the following levels per kilogram of diet: thiamine hydrochloride, 5.0 mg; riboflavin, 10.0 mg; pyridoxine, 5.0 mg; nicotinic acid, 100 mg; calcium pantothenate, 100 mg; choline chloride, 200 mg; inositol, 200 mg; p-aminobenzoic acid, 100 mg; alpha tocopherol, 100 mg; 2-methyl-1:4-naphthoquinone, 50.0 mg; ascorbic acid, 100 mg, and ethyl linolate, 10 g. To this composition, sulfaguanidine was added at 1 and 2% levels, replacing an equivalent amount of sucrose. All results obtained applied equally to 1 and 2% levels of the sulfonamide.

Rats on the above diet, lacking only biotin and "folic acid," show a retarded growth rate. At a 2% sulfaguanidine level, 14 out of 73 rats have survived for 5 months on the diet, and the weights average 180 g for the males and 100 g for the females. More rats survive at the 1% sulfaguanidine level (20 out of 70), but the growth rate does not differ materially from that of the rats on the 2% level.

Rats given the basal diet and supplements described above but receiving 1 μg of biotin methyl ester daily show both a greater survival and improved growth rate. At the 2% sulfaguanidine level, 22 out of 50 rats have survived for 5 months, the weights averaging 220 g for the males and 160 g for the females. Forty out of 50 rats have survived on the 1% sulfaguanidine level, and the weights were not significantly different from those on a 2% level. These observations confirm the findings of Daft *et al.*[11] and Nielson and Elvehjem[12] demonstrating a growth-promoting action of biotin in rats on sulfonamide containing diets and were arrived at independently.

In all of these sets containing 1 and 2% sulfaguanidine, with or without biotin, the rats showed a marked greying at 5 months. This is a generally symmetrical type of greying: the rats at first look dusty and then silvery in color. The extent is equal to that seen in rats on diets deficient in pantothenic acid.

In an attempt to determine the nature of the factor still lacking in the diet containing biotin and all other known vitamins, various supplements were given. Both yeast and liver (0.5 and 0.25 g daily) caused a sharp increase in growth rate equal to 30 g in the first week and continuing until a normal rate comparable to that seen in rats on stock diets was obtained. The greying was completely cured by these supplements in one to 2 months. It was apparent that the "folic acid" of Snell and Peterson[13] was the only known factor missing. Concentrates of this factor were prepared according to the procedure of Hutchings, Bohonos and Peterson.[14] Rats given 3 mg of this concentrate per day showed growth gains equal to that seen with liver and yeast, namely 30 g in the first week. Adequate concentrate was available to keep 5 rats on this supplement for a period of one month, during which time growth rate was restored to normal and the greying of the fur was cured completely in 3 rats and partially in the other 2. Again, this observation reached independently confirms that of Nielson and Elvehjem[12] on the growth effect of "folic acid." The observations on the chromotrichial action of "folic acid" have not been reported.

Supplements of p-aminobenzoic acid (3 mg daily) given to rats on diets containing sulfonamides and all known factors, including biotin but not "folic acid," result in growth gains but these are interpreted as being due to the anti-sulfonamide action of this aromatic amine rather than to a vitamin-like action. Additional supplements of calcium pantothenate (1 mg) cause no growth

[11] Daft, F. S., Ashburn, L. L., and Sebrell, W. H., *Science*, 1942, **96**, 321.

[12] Nielson, E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **145**, 713.

[13] Snell, E. E., and Peterson, W. H., *J. Bact.*, 1940, **39**, 273.

[14] Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, 1941, **141**, 521.

response and no alteration in the color of the fur.

Rats maintained for 5 months on a diet containing 2% sulfaguanidine showed blood levels of 1.75 mg per 100 cc. This value, average for 10 rats, is included to indicate the fact that even at 2% levels, sulfaguanidine does not reach toxic levels in the diet.

Study of the synthesis of factors in the tract or of the synthesis of factors by bacteria normally present in the tract has now demonstrated the synthesis of biotin,[12] "folic acid,"[12] vitamin K,[16] riboflavin,[17] thiamine,[1] nicotinic acid,[17] inositol[18] and pantothenic acid.[19] The material herein reported adds to the importance of the concept of symbiotic relationship of the bacteria of the intestinal tract to the body. The existence of grey fur on rats receiving adequate amounts of p-aminobenzoic acid and calcium pantothenate has been reported.[15] In this communication,[15] it was suggested that p-aminobenzoic acid played its major role in altering the flora of the intestinal tract. An observation common to most physicians is that grey hair frequently occurs following a protracted illness associated with gastro-intestinal disease. The entire picture indicates the importance of bacterial flora in achromotrichia.

The problem of nutritional achromotrichia assumes a less controversial aspect with the knowledge of the role of "folic acid." As it is impossible to produce a "folic acid" deficiency in the rat without the use of sulfonamides to reduce the intestinal synthesis of this factor, this type of achromotrichia could only be produced without sulfonamides if vitamin balance were altered in a manner to affect the composition of the intestinal flora, thus altering the synthesis of "folic acid." This involvement of a third factor, "folic acid," in nutritional achromotrichia affords a simple explanation for the discordant results reported on the role of p-aminobenzoic acid in achromotrichia. Several investigators[20–22] have noted some effect from p-aminobenzoic acid; others[23–25] have seen no effect. It is our conclusion that p-aminobenzoic acid plays a role in nutritional achromotrichia only in so far as it alters the intestinal flora and by so doing alters the intestinal synthesis of "folic acid."

Summary. Biotin and "folic acid" are growth factors for rats on synthetic diets containing sulfaguanidine. "Folic acid" is also a chromotrichial factor for these rats.

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16 Ansbacher, S., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 421.

17 Burkholder, P. R., and McVeigh, I., *Proc. Nat. Acad. Sci. U. S.*, 1942, **28**, 285.

18 Woolley, D. W., *J. Exp. Med.*, 1942, **75**, 277.

19 Henderson, L. M., McIntire, J. M., Waisman, H. A., and Elvehjem, C. A., *J. Nutr.*, 1942, **23**, 47.

20 Ansbacher, S., *Science*, 1941, **93**, 164.

21 Martin, G. J., *Am. J. Physiol.*, 1942, **136**, 124.

22 Pfaltz, H., *Z. f. Vit. Forsch.*, 1942, **12**, 193.

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25 Gyorgy, P., and Poling, C. E., quoted in *Ann. Rev. Biochem.*, 1942, **11**, 340.

13973

Effect of Heat Sterilization on Growth-Promoting Activity of Pyridoxine for *Streptococcus lactis* R.

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In a previous publication[1] the existence of a physiologically active metabolite of pyridoxine which possessed much greater activity for *Streptococcus lactis* R than did synthetic pyridoxine itself was demonstrated. For convenience this metabolite (or mixture of metabolites) was called "pseudopyridoxine." It was further shown that the absorption of pyridoxine by this organism from media in which pyridoxine (or a derivative) was in limiting concentration did not occur to a measurable extent. This result is in contrast to those secured with all other known nutrilites which have been investigated. On the basis of this fact, it was postulated that pseudopyridoxine, rather than pyridoxine, was the physiologically essential factor for this organism. The growth-promoting activity of pyridoxine, according to this view, resulted from a transformation of a minute amount of pyridoxine to a more highly active form (pseudopyridoxine), which was then utilized by the organism for growth, while most of the pyridoxine present remained unchanged and hence unavailable for growth of the organism. It was not clear why such a conversion process should cease, and growth of the organism stop, while an excess of unchanged pyridoxine was still present in the medium.

Results cited below show that the conversion results not from activities of the test organism, but rather from interaction of pyridoxine with certain constituents of the medium during heat sterilization. It is shown that the activity of pyridoxine can vary greatly, depending upon how long it is autoclaved with the medium.

TABLE I.
Effect of Autoclaving* with Medium on Growth-Promoting Action of Pyridoxine.

Pyridoxine hydrochloride added, γ per 10 cc	Galvanometer reading†			
	Unheated	Autoclaved 10 min.	Autoclaved 20 min.	Autoclaved 40 min.
0	10	10	10	10
0.2		18	37	43
0.4		26	46	54
0.7	12	35	54	54
1.0	15	42	54	
2.0	15	53		
3.0	21	55		
5.0	29			
10	34			
30	43			
Ratio of activities	1.0	13	41	65

*The culture tubes were autoclaved at 15 lb steam pressure.

†This is a measure of culture turbidity: a reading of zero indicates 100% transmission; a reading of 100 is complete opacity.

Experimental. The medium and technic used were exactly similar to those previously described in detail.[1]

Results. The comparative activity of given amounts of pyridoxine in promoting growth of *Streptococcus lactis* after various periods of autoclaving with the complete medium is shown in Table I. The most

[1] Snell, E. E., Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, 1942, **143**, 519.

turbid culture obtained gave a galvanometer reading of 55; to achieve this degree of growth required more than 30γ of pyridoxine in the unheated test, whereas only 2, 0.7 and 0.42γ were required to produce a like amount of growth after autoclaving for 10, 20, and 40 min, respectively. The ratio of growth-promoting activity after a given period of autoclaving to that of unheated pyridoxine is given in the last line of the table. These values were calculated from the standard curve obtained after 10 min of autoclaving[1] and demonstrate the profound effect of autoclaving with the medium on the growth-promoting power of pyridoxine for this organism.

Tests were next made to determine which constituents of the medium were responsible for this effect. It was found that autoclaving pyridoxine at pH 7.2 with the hydrolyzed casein of the medium, or with the cystine or tryptophane alone, was effective in increasing its activity. Autoclaving under the same conditions with any of the other constituents of the medium had no such effect. Thus the effect appeared due to interaction of some type between pyridoxine and amino acids during autoclaving. Individual amino acids were therefore compared as to their effectiveness in producing this change. 0.1 mg portions of pyridoxine hydrochloride were added to 10 cc of a solution which contained 50 mg of sodium acetate plus 1 to 5 mg of various amino acids. The pH was 7.2. All tubes were autoclaved at 15 lb pressure for 30 min. The contents of each tube were then assayed with *S. lactis* for their apparent pyridoxine content. The entire assay was autoclaved 10 min as previously described.[1] Results are given in Table II. The effect is quite non-specific, since it is given by each of the amino acids tested. Cystine was especially effective; the next most effective amino acid was glycine. The effect of varying the concentrations of cystine and glycine is shown in Table III. Again, cystine is the more effective in producing the activation.

Discussion. These observations indicate that synthetic pyridoxine is almost, if not completely inactive as a growth factor for *S. lactis*. It becomes active due to formation in minute amounts of a substance of unknown structure when pyridoxine is heated with the amino acids of the medium during sterilization. The inactivity of unchanged pyridoxine in promoting growth of this organism explains the previously observed fact that growth ceased in a medium where growth apparently was limited only by the pyridoxine concentration before appreciable amounts of pyridoxine were absorbed. It was shown[1] that a derivative of pyridoxine of similarly high activity for *S. lactis* occurs universally in natural extracts, and that pyridoxine is partially converted to a product

TABLE II.
Effect of Autoclaving Pyridoxine with Individual Amino Acids on Its Growth-Promoting Activity.

Amino acid	Amt, mg per 10 cc	Activity compared with untreated pyridoxine*
None		1.1
l-Tryptophane	1.0	1.8
l-Cystine	1.0	5.2
dl-Alanine	5.0	2.3
Glycine	5.0	3.9
l-Lysine	5.0	1.9
d-Arginine	5.0	2.6
l-Histidine	5.0	1.3
dl-Phenylalanine	5.0	2.6
dl-Leucine	5.0	2.2
Casein Hydrolysate†	5.0	2.1

*These comparisons were made in an assay where all tubes were autoclaved for 10 minutes. This increase in activity therefore represents that which occurred as a result of the above treatments *in addition* to that occurring during 10 minutes of autoclaving with the medium, as shown in Table I.

†Norite-treated: assay of any of the above products alone at dilutions present in the test show an apparent pyridoxine content of zero.

TABLE III.
Effect of Autoclaving with Various Concentrations of Cystine and Glycine on Activity of Pyridoxine.

Amino acid	Amt. mg per 10 cc	Activity compared to untreated pyridoxine*
None		1.1
l-Cystine	0.10	1.5
,,	0.30	2.7
,,	1.00	5.9
,,	3.00	8.2
,,	10.00	10.6
Glycine	0.10	1.3
,,	0.30	1.4
,,	1.00	2.5
,,	3.00	4.2
,,	10.00	4.1

**Cf.* footnote to Table II.

of similarly high activity by animal passage. These conclusions stand unaltered by the present findings. Whether the substances present or formed in these different instances are identical, remains to be demonstrated. Activation of pyridoxine in this manner by heating *in vitro* is not an effect of amino acids alone. Such substances as thioglycollic acid, ammonia, and others also promote the change.

One other strain of *S. lactis* tested behaves the same as that used in this study. Yeast is not dependent upon such a change in the pyridoxine molecule before it is able to use it. Other organisms have not been studied in this regard. Landy and Dicken[2] have recently recommended *Lactobacillus casei* as a test organism for the assay of pyridoxine in natural materials. In our experience, this assay is subject, in a lessened degree, to the same disturbing influences previously outlined[1] and those mentioned above. For example, in one assay carried out according to their procedure, Difco yeast extract contained 2.0, 0.6 or 0.35 mg of pyridoxine per gram as determined after autoclaving the test for 10, 20 or 40 min, respectively. Yeast assay[3] gives 0.015 mg pyridoxine per gram of this material. It is evident that much more investigation into forms of pyridoxine which are physiologically active for animals is required before any organism can be used routinely for the estimation of "vitamin B_6" in natural materials.

Summary. Synthetic pyridoxine is almost inactive as a growth factor for *S. lactis* R in a pyridoxine-free medium if heat sterilization is avoided. Autoclaving media which contain pyridoxine greatly increases its activity for this organism. The same effect was achieved in varying degrees by autoclaving pyridoxine at neutrality with individual amino acids. Cystine and glycine were most effective in producing this change.

2 Landy, M., and Dicken, D. M., *J. Lab. Clin. Med.*, 1942, **27**, 1086.

3 Williams, R. J., Eakin, R. E., and McMahan, J. R., *Univ. Texas Pub.*, 1941, No. 4137, 24.

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Biotin Deficiency in the Rat.

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A number of investigators have reported the occurrence of depigmentation of the pelage of rats maintained on egg-white containing rations. György and co-workers[1,2] noted a brownish discoloration of the black fur, or single gray hairs interspersed in the fur without generalized graying, or both. Sullivan and Nicholls[3] also observed a diffuse, partially decreased pigmentation in some animals receiving egg-white containing rations. Symmetric patterns of grayness such as produced by a deficiency of pantothenic acid were not observed. In the present study the occurrence of a symmetrical achromotrichia is described in black rats maintained on dried, fresh egg-white containing rations after curative therapy had been initiated by the feeding of biotin. The pattern was the reverse of that observed in pantothenic acid deficiency.

A comparison was made of several diets in respect to the production of the biotin deficiency syndrome and the alleviation of symptoms with biotin therapy.

Experimental. Littermate weanling male rats were segregated into each of 6 dietary

1 György, P., in Pfaundler, A., and Schlossmann, M. v., *Handbuch der Kinderheilkunde*, 1935, **10**, 55.

2 György, P., and Poling, C. E., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 773.

3 Sullivan, M., and Nicholls, J., *Arch. Derm. and Syph.*, 1942, **45**, 295.

TABLE I.
Composition of Diets.

Ingredients in diet	G per 100 g					
	19	20	21	22	23	24
Egg-white*	50	50	30	30	15	15
Casein (Harris)	—	—	—	—	15	15
Sucrose	34	24	54	44	52	54
Crisco	10	10	10	10	10	10
Yeast (brewers')	—	10	—	10	—	—
Salts No. 4	4	4	4	4	4	4
Cod liver oil	2	2	2	2	2	2
Dried whole liver	—	—	—	—	2	—

Addenda Diets: 19, 21, 23 and 24.

	Mg per 100 g
Thiamin	0.8
Riboflavin	1.6
Pyridoxine	0.8
Ca pantothenate	5.0
Nicotinamide	10.0
Choline Chloride	100.0

*Dried fresh flake—Henningsen Bros., 60 Hudson Street, New York City.

groups (10 animals in each lot).

The diets were shown in Table I.

The first evidence of biotin deficiency (loss of the guard hairs leaving a soft, downy mole-like coat) was noted in all animals after 3-4 weeks on diet. An exfoliative dermatitis of the abdomen followed, progressing to a more or less general alopecia. The "spectacle eye" was observed in a large percentage of the animals, particularly those maintained on diets 23 and 24. The abnormality in gait, typical of the deficiency, was seen in 8 of the rats and again 6 of the 8 cases were with the animals receiving diets 23 and 24. This abnormality of locomotion, a type of hopping gait, developed within 4 weeks. Nielson and Elvehjem[4] noted this type of gait only after their animals, on 10% egg-white containing rations, had been on experiment for 9-12 weeks. These workers were able to produce this condition in 9 weeks only if the diets were low in fat. The presence of high amounts of fat in their diets slightly delayed the onset of the paralysis.

As is seen in Table II, the most satisfactory growth occurred with the group of rats receiving 15% egg-white and 15% casein but

TABLE II.
Gain in Weight of Rats After 40 Days on Egg-white Containing Diets.

Diet	Gain in wt, g
19	94
20	125
21	105
22	110
23	147
24	116

with 2% of dried liver incorporated in the ration (diet 23). The rapid growth of the animals may account for the development of a more complete deficiency picture. The apparent superior growth of the rats receiving diet 20 is deceptive in that 4 of the initial 10 rats died during the second or third week of the experiment.

After approximately 35 days on test, the weights plateaued for the several groups and supplementation with crystalline biotin was initiated. The rats receiving 50% and 30% egg-white were segregated into 2 like groups (as judged from weight and severity of the dermatitis etc.) and were dosed orally by stomach tube with 1 or 2 μg of free biotin 6 times weekly. The animals on the 15% egg-white diets were given 0.5 or 1 μg. After 3 days of supplementation a marked improvement was evident; the heretofore erythematous areas were blanched and the animals were clean in appearance. The "washboard"-like scaly dermatitis of the abdomen had largely disappeared. The abnormality in gait was no longer evident after one week of therapy; a finding in agreement with that of Sullivan and co-workers[5] who found that rats likewise affected were relaxed and assumed a normal posture and gait after 3 or 4 days of biotin treatment. These investigators were unable to find any anatomical change in the central nervous system. They suggested that the hypertonicity might be due to irritability and that the rigidity was not myotonic in origin. Nielson and Elvehjem[4] noted a complete cure after 3 to 4 weeks of biotin therapy. A marked growth response was noted in all animals.

[4] Nielson, E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **144**, 405.

[5] Sullivan, M., Kolb, L., and Nicholls, J., *Bull. Johns Hopkins Hosp.*, 1942, **70**, 177.

TABLE III.
Growth Response to Biotin.

Biotin 6 times weekly	No. of rats	Diet	Wt gain 30 days, g
1	5	19	79
2	5	19	74
1	3	20	74
2	3	20	66
1	4	21	44
2	4	21	74
1	5	22	54
2	4	22	72
0.5	4	23	53
1	4	23	64
0.5	4	24	54
1	5	24	68

The average weight gains are shown in Table III.

The quantities of biotin fed were, in all probability, sub-optimal since improvement was not continuous *e.g.*, the spectacle eye condition persisted in some animals after 5 weeks of biotin therapy. Employing different diets, Nielsen and Elvehjem[6] produced a complete regression of this condition after 2-3 weeks of biotin administration.

The most striking observation was in the

[6] Nielsen, E., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 349.

FIG. 1.
Lateral graying of the pelage. Biotin-depleted rat—fed biotin.

FIG. 2.
Diamond shaped depigmented area on head. Biotin-depleted rat—fed biotin.

pelage, for 29 out of 30 black rats, irrespective of diet, showed a symmetrical graying of the ingrowing fur, after 12 days of supplementation. The gray pattern outlined the lateral aspect of the head and trunk and in most cases extended from the tip of the nose to the base of the tail (Fig. 1). A graying or rusting of a triangular or diamond-shaped area on the top of the head was observed in several cases (Fig. 2). It is of interest to note that the distribution of the pattern is the reverse of that observed in rats deprived of, or receiving sub-optimal intakes of pantothenic acid. The graying persisted to some extent even after 5 weeks of therapy and was not infrequently accompanied by rusting.

Summary. It is possible to reduce materially the time necessary to produce in the rat a fully, developed syndrome of biotin deficiency by feeding a diet containing in addition to 15% dried egg-white and 15% casein, 2% dried liver. On this diet most of the signs develop within less than 4 weeks.

Biotin depleted animals fed small doses of biotin curatively showed a characteristic, symmetrical lateral graying of the pelage that persisted to some extent even following 5 weeks of biotin supplementation.

We wish to thank Mrs. Elsa Zitcer and Miss Elizabeth Wurtz for their valuable technical assistance.

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The Proteus X Bacilli and the Weil-Felix Reaction.

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The probable existence of typhus fever of the endemic type in Puerto Rico[1,2] has recently aroused much interest among the medical profession concerning the reliability of the Weil-Felix reaction, under local conditions, in the diagnosis of this disease.

During the course of an investigation of the proteus group of bacilli, 72 cultures of proteus X organisms utilized in the diagnosis of certain rickettsial diseases, kindly supplied to us by 30 Public Health laboratories in the United States, have been studied.

Sixty-seven out of the 72 cultures investigated gave typical biological and agglutination reactions, but 5 cultures reacted atypically in one way or another.

The carbohydrates (20% aqueous solutions) were sterilized by filtration and added to the sterile medium (Difco's phenol red broth base) to obtain a final concentration of approximately 1%. For the production of H_2S, indol and gelatin liquefaction, lead acetate agar, tryptophane broth and nutrient gelatin (all Difco products) respectively were used. Beef extract broth to which methylene blue was added to give a final concentration of 1:5000 was utilized in testing for the reduction of this dye. Rabbits were immunized by intravenous injection of fresh living suspensions.

Fermentation and other reactions, except indol, were read after 18 hr, 48 hr and one week. The production of indol was tested for after 48 hr. Alcohol-treated suspensions were used in all the agglutination tests.[3]

No difference was noted in the action on gelatin among the different cultures of X type bacilli studied. It was interesting to note the complete absence of agglutination, with a known typhus serum of high titer, of 21 out of the 23 proteus X-2 cultures, and the slight agglutination of 3 out of the 14 proteus XK cultures tested.

All the X-2 and X-19 cultures fermented saccharose, maltose, dextrose, salicin and galactose with the production of acid and gas and xylose and glycerol with the production

[1] Pons, J. A., *Bol. Asoc. Méd. de P. R.*, 1940, **32**, 196.

[2] Riera López, S., Watt, J., and Doull, J. A., *P. R. Jour. Pub. Health and Trop. Med.*, 1942, **17**, 216.

TABLE I.

Agglutination Reactions with Alcohol-Treated Suspensions of Proteus X Organisms (X-19, OX-19, X-2 and OXK) with Sera of Normal Individuals and of Hospital Patients Suffering of Conditions Other Than Typhus.

Antigen	Type of cases	Total No. of cases	1:25	1:50	1:100	1:200	1:400	Negative %
OXK	Normal Americans*	315	76.2‡	18.2	1.2	0.6	0	23.8
	Normal Puerto Ricans	211	81.8	19.8	2.83	0.5	0	18.2
	Patients†	268	81.3	26.8	5.9	2.2	0.7	18.0
X-2	Normal Americans	344	50.2	27.1	8.3	3.1	0.9	49.7
	Normal Puerto Ricans	211	60.2	28.0	11.9	3.8	2.4	39.8
	Patients	259	63.9	40.1	20.8	9.3	0.8	35.8
OX-19	Normal Americans	413	45.0	22.4	9.6	1.7	0.5	55.9
	Normal Puerto Ricans	266	39.0	20.0	5.7	0.4	0	60.1
	Patients	265	42.5	21.8	6.4	2.6	1.1	57.3
X-19	Normal Americans	414	87.2	73.2	49.1	28.4	14.2	13.7
	Normal Puerto Ricans	263	84.4	66.8	45.9	22.8	8.0	15.6
	Patients	372	79.8	66.4	42.5	21.2	8.4	20.2

*Soldiers, sailors and civilians coming to the blood bank at the School of Tropical Medicine in San Juan.

†Hospital and clinic patients (Puerto Ricans) of the University Hospital in San Juan.

‡Figures refer to percentage of cases giving agglutination with the corresponding serum dilution.

of acid and gas or acid alone. Mannite, lactose, sorbitol, dulcitol, raffinose and dextrin were not fermented. The action on levulose was variable. Methylene blue was reduced; hydrogen sulfide and indol were produced.

The XK cultures did not ferment either maltose or salicin and did not produce indol; otherwise they behaved as the X-2 and X-19 cultures.

Among the atypical cultures, 3 gave atypical biological reactions but retained their typical agglutinating properties. One of the atypical cultures (supposedly X-19) did not agglutinate with the rabbit antisera (anti-X-19, anti-X-2, anti-XK) or with known typhus sera, but gave the typical biological reactions. Another culture fermented salicin and maltose but did not form indol.

Approximately one thousand persons, 700 normal and 300 clinic or hospital patients, suffering from conditions other than typhus, were tested for the presence of agglutinins in their blood against alcohol-treated suspensions[3] of proteus OXK, X-2, OX-19, and X-19. The results are summarized in Table I.

[3] Wadsworth, A. B., *Standard Methods*, p. 518, The Williams and Wilkins Company, Baltimore, 1939.

It is interesting to note the large number of sera which agglutinated proteus OXK in 1:25 dilution and the marked difference in the proportion of sera containing agglutinins against OX-19 as compared with X-19 antigen, especially in the higher dilutions. The fact that the sera of many persons, normal and patients alike, agglutinated the X-2 antigen must be also noted.

It is interesting to observe that there was no appreciable difference in the proportion of cases whose sera agglutinated the different antigens, among the normal Americans, normal Puerto Ricans and Puerto Rican patients.

Summary and Conclusions. 1. Out of the 72 proteus X cultures studied, 5 gave atypical reactions. One of the atypical cultures, presumably X-19, gave the typical biological reactions, but failed to agglutinate with high titer typhus serum and with anti-OX-19, X-2, X-19 and OXK rabbit-immune serum.

2. The non-liquefaction of gelatin cannot be used as a criterion in the differentiation of members of proteus X bacilli.

3. One of the atypical X-19 cultures (No. 68) has lost the power to form indol but has retained the ability to ferment maltose and salicin and to agglutinate with anti-X-19

rabbit serum and typhus serum.

4. A positive agglutination in 1:400 serum dilution is significant, when the OX-19 strain supplied by the National Institute of Health is utilized. The X-19 strain must not be used even when the suspensions are treated with phenol and with alcohol, because it gives apparently non-specific agglutination in relatively high dilution with the sera of many healthy persons.

5. The serum of a large proportion of persons agglutinated proteus OXK organisms in 1:25 dilution. It was observed that the agglutination was strong, usually 3 plus, in 1:25 dilution and completely negative or very weak in 1:50 dilution in the majority of the sera.

6. The vast majority of the X-2 cultures tested (20 out of 23) failed to agglutinate with high titer typhus serum. This is not in accordance with the statement frequently encountered in the literature and in textbooks that X-2 organisms are agglutinated by typhus serum.

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Influence of Fat Mobilization on Acetone Body Production.

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The administration of estrogens to male or female birds results in a rapid increase in the blood lipids,[1-3] which is associated with the infiltration of large amounts of fat into the liver. When the administration of the estrogens is discontinued, there is a rapid return of the blood lipids to the normal concentration.

In view of these facts, it became of interest to study the influence of the rapid mobilization of fat on the rate of ketogenesis. Two groups of ducks were fasted for 3 days, and then one group was given daily a subcutaneous injection of 4 mg diethyl stilbesterol suspended in sesame oil, while the other group, used for control purposes, received a daily injection of 0.8 cc peanut oil. Every third day thereafter, until the 18th day after the beginning of the fast, blood samples were drawn for the determination of the concentration of fat[4] and total acetone bodies.[5]

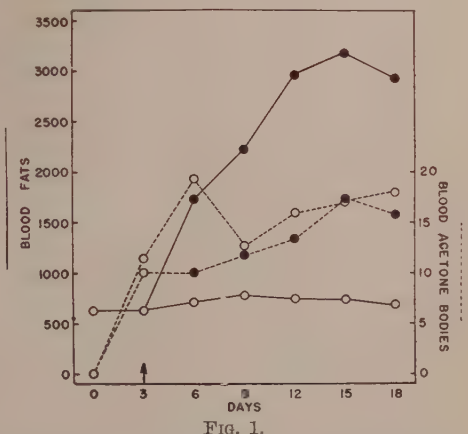

FIG. 1.

The influence of stilbesterol on blood total lipids and total acetone bodies. The open circles refer to animals receiving peanut oil and the black circles refer to animals receiving stilbesterol. The injections began on the third day after the beginning of the fast. The dotted lines depict the blood total acetone bodies, the solid lines the blood total lipids.

The results are depicted in Fig. 1, where each point represents the average findings from 6 ducks. Although a rapid lipemia ensued in consequence of the administration of stilbesterol, there was no significant effect on the rate of acetone body accumulation in

1 Zondek, B., and Marx, L., *Nature*, London, 1939, **143**, 378.

2 Lorenz, F. W., Chaikoff, I. L., and Entenman, C., *J. Biol. Chem.*, 1938, **126**, 763.

3 Flock, E. V., and Bollman, J. L., *Proc. Staff Meetings of the Mayo Clinic*, 1941, **16**, 783.

4 Street, H. R., *J. Biol. Chem.*, 1936, **116**, 25.

5 Mirsky, I. A., Nelson, N., and Grayman, I., *J. Biol. Chem.*, 1939, **130**, 179.

the blood. If anything, the stilbesterol-treated ducks showed a lower rate of acetone body accumulation than did the control ducks.

It is now recognized by many that the acetone bodies are the products of normal fat oxidation in the liver.[6,7] It has been demonstrated also that an infiltration of fat in the liver is associated with the lipemia produced by stilbesterol injections,[2,3] presumably in consequence of an extensive mobilization of fat from the depots to the blood stream. Accordingly, if the availability of lipid substrate were a factor in the oxidation of fat in the liver, an increase in the rate of acetone body production would ensue in consequence of stilbesterol administration. Our data indicate that the latter does not occur. Hence, they support the hypothesis that the availability of fats and their oxidation in the liver are two independent phenomena and that an increase in the former does not influence the latter.

Summary. The administration of stilbesterol produces a marked lipemia but does not influence the rate of acetone body production.

6 Mirsky, I. A., *J. A. M. A.*, 1942, **118**, 690.

7 MacKay, E., *Proc. Am. Diab. Assn.*, 1942, **2**, 135.

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Changes in Blood and Urine after Intravenous Amino Acid Mixture in Patients with Liver Disease.*

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Recent reports of studies on patients show that mixtures of the essential amino acids, as obtained by enzymatic digestion of casein, can be used to maintain nitrogen balance when given intravenously.[1-6] The statement has been made, however, that in patients with liver disease the method is contraindicated. Our clinical experience does not support this conclusion and, although the question requires further study, the data acquired in the present experiments seem to have some relevancy.

Experimental Method. The solution of amino acids used ("Amigen") was supplied by Dr. Warren M. Cox, Jr. of Mead Johnson and Company, and consisted of a sterilized aqueous nonpyrogenic 10% solution of an enzymatic hydrolysate of purified casein and pork pancreas. The nitrogen of the solution is present chiefly as nitrogen of amino acids and polypeptides, and the material is not allergenic. The solution contains the amino acids essential for maintenance and growth as shown by animal experiments. The hydrogen ion concentration of the solution was adjusted to a pH value of 6.5.

Amino acid concentration in whole blood and plasma was determined by the ninhydrin-CO_2 method recently developed by Van Slyke, MacFadyen and Hamilton.[7] Amino acid concentration in urine was likewise determined by the ninhydrin-CO_2 technic devised by the same investigators but as yet unpublished.[8] Standard methods were

* Aided by a grant from Mead Johnson and Company.

1 Elman, R., and Weiner, D. O., *J. A. M. A.*, 1939, **112**, 797.

2 Shohl, A. T., Butler, A. M., Blackfan, K. D., and MacLachlan, E., *J. Pediat.*, 1939, **15**, 469.

3 Farr, L. E., and MacFadyen, D. A., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 444.

4 Elman, R., *Ann. Surg.*, 1940, **112**, 594.

5 Brunschwig, A., Clark, D. E., and Corbin, N., *Ann. Surg.*, 1942, **115**, 1091.

6 Landesman, R., and Weinstein, V. A., *Surg., Gyn. and Obstet.*, 1942, **75**, 300.

7 Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P., *Fed. Proc.*, 1942, **1**, 139.

TABLE I.

Plasma and Urine Changes During Amino Acid Infusion.

Between samples 1 and 2, 1000 cc 10% amino acids solution (Amigen) was given intravenously in 5 to 6 hours. Sample 3 was taken 24 hours after sample 1. The patients were fasting but took water as desired.

Case	Sample	Plasma			Urine		
		HCO_3, vol/100 cc	Amino N, mg/100 cc	NPN, mg/100 cc	Vol., cc	Amino N, mg	Total N, mg
1. ♀ Toxic hepatitis, liver failure	1	58.7	7.2	22.6	168	9	910
	2	49.4	9.0	32.8	403	69	3,295
	3				1,030	102	9,600
2. ♀ Obstructive jaundice, stone	1	58.1	8.0	13.9	220	17	482
	2	50.3	13.5	35.5	430	128	1,660
	3	57.3	5.7	23.1	870	86	6,870
3. ♀ Same	1	60.5	7.3	34.0	103	9	427
	2	54.7	10.7	45.9	665	70	3,030
	3	51.0	9.5	36.8	865	62	5,280
4. ♀ Cirrhosis, liver failure	1	75.5	6.3	25.3	30	11	322
	2	70.0	14.3	49.3	285	236	2,840
	3	69.2	6.1	36.1	345	158	5,450
5. ♂ Obstructive jaundice, liver failure	1	85.5	5.1	47.0	*		
	2	73.8	13.7	74.7	330	96	6,130
	3	71.3	3.1	73.7	675	95	12,530
6 ♀ Toxic hepatitis, melena, anemia	1	62.0	5.0	16.2	*		
	2	54.7	10.5	42.0	860	104	3,370
	3	55.2	4.3	23.9	1,230	28	5,920
7. ♂ Herniorraphy, convalescent	1	71.3	7.6	24.6	*		
	2	63.6	12.4	46.8	545	66	4,380
	3	69.0	4.9	34.0	1,445	92	11,345
8. ♂ Echinococcus cyst of liver, not jaundiced	1	65.3	5.2	21.4	*		
	2	59.8	8.7	61.5	645	132	3,795
	3	50.7	3.8	34.7	1,510	65	6,820

*Discarded.

used in the other analyses.

The patients were kept under observation constantly during the study and in no case was it necessary to discontinue the infusion on account of untoward symptoms. The infusions were given through arm veins by steady drip, the rate being kept as nearly as possible the same in comparable experiments. Urine was collected without loss by means of an indwelling urethral catheter in the experiments of Table II, while in the other cases the patients were able to void at stated intervals. The patients had fasted overnight only. Water was drunk as desired during the experiments but no food or drugs were taken.

Presentation of data. Table I is based on studies of patients who received 1000 cc 10% amino acid solution intravenously at a fairly constant rate, between 5 and 6 hr being required for the infusion. Sample No. 1 was taken at the beginning of the infusion, sample No. 2 at the end. Sample No. 3 was obtained 24 hr after the first sample, while the patient was still fasting. Patients 1, 2, 3, 4, 5 and 6 had severe liver disease, while patients 7 and 8 had no demonstrable disturbance in liver or kidney function and hence served for control observations.

In Table II are shown changes in plasma and urine resulting from giving amino acids at a rapidly increasing rate intravenously without increasing, however, the volume of fluid infused. Blood samples were taken at the beginning and at the end of the infusion. In the final period urine was collected but

[8] Personal communication from Dr. D. D. Van Slyke.

TABLE II.
Effects of Amino Acids Infusion in Patient Z, Female, Toxic Hepatitis and Liver Failure (Case 1) and in Patient C, Female, Possible Biliary Dyskinesia (Case 2).

		Infusion			Urine			Plasma	
Case	Period	Sol., quantity	Duration, min.	Tot. N, mg	Vol., cc	Tot. N, mg	Amino N, mg	Amino N, — Tot. N	Amino N, mg/100 cc	NPN, mg/100 cc
1	1	300 cc 5% glucose	85	0	54	340	9	2.6%	3.8	14.6
	2	" { 2.5% amigen, 7.5% glucose	110	900	95	880	19	2.1		
	3	" { 5% amigen, 5% glucose	110	1,800	115	1,130	30	2.6		
	4	" 10% amigen	125	3,600	173	1,595	52	3.3	7.6	19.3
	5	–	135	0	100	991	23	2.3		
	Total		565	6,300	537	4,936	133			
2	1	300 cc 5% glucose	90	0	240	547	5	0.9%	5.8	21.9
	2	" { 2.5% amigen, 5% glucose	110	900	137	415	4	1.1		
	3	" { 5% amigen, 5% glucose	110	1,800	217	653	17	2.6		
	4	" 10% amigen	110	3,600	145	547	19	3.5	11.2	29.8
	5		150	0	650	1,832	28	1.5		
	Total		570	6,300	1,389	3,994	73			

The rate of intravenous administration of amino acids was progressively increased, but the volume of fluid infused was not increased. There was no interval between the different infusions. The patients were fasting but were allowed to drink small amounts of water as desired. Urine was collected without loss by means of an indwelling catheter.

no fluid was administered intravenously. Except during the first period the fluid given was hypertonic as compared with plasma, and slightly acid in reaction. In Case 1 severe liver disease was present. In Case 2 liver and kidney function tests were normal.

Comments. In these experiments the urinary nitrogen data have little significance with respect to nitrogen balance, for the patients had fasted only overnight beforehand. The experimental plan was not designed to show the extent of utilization of amino acids injected in this manner. It is of interest, however, that only trivial amounts of amino acids were excreted in the urine, even during and after excessively rapid administration. The urinary total nitrogen in every case was large, from which it appears that the severely damaged liver still possesses the power to deaminize amino acids at a rapid rate. This conclusion depends on the validity of the method used for determining amino acid nitrogen in the urine. The urea nitrogen was not determined in the urine as such, but presumably this formed the chief component of the total nitrogen excreted. In only one of the 8 patients of Table I was the non-protein nitrogen of the plasma elevated beyond the normal range 18 hr after the amino acid infusion. Evidently the power of the kidneys to excrete nitrogen was adequate, even in patients showing signs of liver failure, such as jaundice, ascites and anasarca.

The rapid infusion of 100 gm of amino acids resulted in a doubling of the plasma amino acid concentration, but the increase was transitory and 18 hr later the value was usually less than the original. This may be interpreted as indicating that amino acids are freely and rapidly diffusible into the tissue fluids, and a large volume of fluid, presumably including intracellular fluid, would be required for the even dilution of the quantities injected in these experiments. The fasting plasma amino acid concentration in 7 observations in the patients with liver disease were similar to the values in 3 patients who showed no evidence of impairment of hepatic function, and the increase following infusion of amino acids was of comparable extent. The amino acid concentration

was as a rule somewhat higher in whole blood than in plasma, both before and after the amino acid infusions. Reduction in plasma bicarbonate concentration occurred invariably as an immediate result of the amino acid infusion, both in the patients with liver disease and those not having hepatic impairment.

When the rate of infusion of amino acids was progressively increased, as shown in Table II, the changes in urine and plasma amino acid concentration are found to be similar in the patient with toxic hepatitis and in the patient without liver disease. The amount of amino acid excreted in the urine in the 2 cases increases somewhat, but remains a small percentage of the total nitrogen excreted. At the end of the period of observation, which lasted 9 hr, there was still a large retention of nitrogen in both cases.

Conclusion. The changes in plasma and urine following infusion of an amino acid mixture were found to be comparable in 2 groups of patients, one group having advanced liver disease and the other showing no evidence of hepatic disability. In neither were untoward clinical effects noted.

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A Study of Gastric Secretion as Influenced by Changes in Duodenal Acidity.

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In spite of numerous investigations there remains considerable difference of opinion regarding the effect of injecting HCl into the intestine on the volume output of gastric juice. Some investigators[2,3] have reported that acid in the intestine may stimulate the flow of gastric juice, others have reported inhibition[1,4,5,7] whereas still others[6] have found that injecting acid into the duodenum appeared to have no significant effect on the volume or acidity of gastric juice secreted in response to a standard meal. Similarly discordant results were obtained in experiments carried out in this laboratory some years ago and the results obtained early in this investigation were likewise inconstant.

This communication is a preliminary report of the results obtained up to the present in our efforts to determine why, on one occasion, the addition of a given volume of HCl to the intestinal contents may profoundly inhibit gastric secretion and may, on another occasion, apparently under the same conditions, have little or no effect.

Two dogs were used each of which was provided with a Pavlov pouch and with a gastric and a duodenal fistula as previously described by one of us.[8,9] The volume of the secretion collected from the Pavlov pouch was measured at 15-min intervals for one hr before and for from 4 to 7 hr after a standard meal consisting of 300 g of beef heart free from visible fat. Thirty-two experiments were performed of which 21 were normal

1 Sokoloff (quoted by Babkin), Thesis, St. Petersberg, 1904.

2 Ivy, A. C., and McIlvain, G. B., *Am. J. Physiol.*, 1923, **63**, 418.

3 Ivy, A. C., Lim, R. K. S., and McCarthy, J. E., *Quart. J. Exp. Physiol.*, 1925, **15**, 55.

4 Day, J. J., and Webster, D. R., *Am. J. Dig. Dis.*, 1935, **2**, 527.

5 Griffiths, W. J., *J. Physiol.*, 1936, **87**, 34.

6 Stevens, R. E., Segal, H. L., and Scott, W. J. M., *Am. J. Dig. Dis.*, 1939, **6**, 706.

7 Shay, H., Gershon-Cohen, J., and Fels, S. S., *Am. J. Dig. Dis.*, 1942, **9**, 124.

8 Thomas, J. E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 58.

9 Thomas, J. E., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 260.

controls in which only the standard meal was administered. In the remainder, N/10 or N/15 hydrochloric acid was introduced into the first part of the duodenum via the duodenal fistula at a constant rate during various periods after the ingestion of the meal. In 9 of the control experiments and in 8 of the experiments with acid, the pH of the duodenal contents, collected approximately 4 inches distal to the point at which the acid was injected, was determined at 15 or 30 min intervals.

The experiments in which the pH of the intestinal contents was measured during the injection of acid revealed what we believe to be the cause of our own previous discordant results and, perhaps, some of those recorded in the literature. We found that regardless of the volume of acid injected into the duodenum, if the pH of the intestinal contents was thereby lowered to 2.5 or below, some degree of inhibition of secretion of gastric juice by the Pavlov pouch was almost invariably observed. If, on the other hand, in spite of the addition of HCl to the intestinal contents, the pH of the duodenal contents remained above this level, the amount of gastric juice secreted by the pouch in response to the standard meal remained within normal limits.

Fig. 1 shows a typical result. The upper curves show the volume in cc of the secretion collected from the Pavlov pouch in one experiment with acid and in a normal control experiment on the same animal. In the experiment recorded by the broken line HCl was injected into the intestine during the first 2 hr after feeding. The results of the control experiment are indicated by the solid line. The corresponding lines in the lower curves show the variations in duodenal pH during the two experiments. Our results as a whole indicate that when the pH of the intestinal contents is depressed to approximately 2.5 by means of injected HCl, the volume of gastric juice secreted by the pouch in response to a standard meal of raw meat is reduced to about one-half that obtained in the control experiments. When the pH of the duodenal contents falls to 2 or below, due to the injected acid, the volume of secretion is frequently less than during the fasting period.

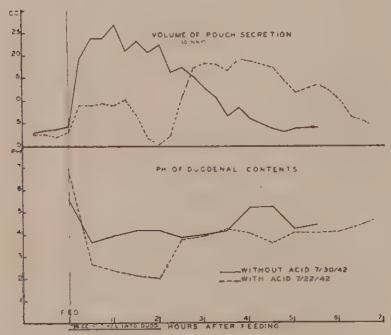

FIG. 1.

Changes in the volume of gastric juice (upper graph—broken line) secreted by a Pavlov pouch in response to a standard meal associated with lowering of the pH of the intestinal contents (lower graph—broken line) to below 2.5 by means of HCl injected into the duodenum. Corresponding data obtained in a normal control experiment (without injecting acid) are also recorded (solid lines).

We wish also to point out the "rebound" or increase in output of pouch secretion following the inhibition produced by acid. In some experiments during this "rebound" phase the volume of pouch secretion reached and maintained levels as high or even higher than were obtained in any control experiments. Considering the large amount of acid that it was necessary to introduce at times in order to depress the duodenal pH sufficiently to cause inhibition of secretion, this "rebound" may have been caused by excess hydration, delayed gastric emptying or other effects of the acid. However, the degree of inhibition preceding the "rebound" appeared to be correlated consistently with the intestinal acidity as measured in pH units and with no other factor that we observed.

Further studies designed to evaluate the effects of hydration, delayed gastric emptying and other incidental effects of the acid injections are at present being carried out and will be reported at a future date.

Conclusions. The introduction of acid into the duodenum inhibits gastric secretion only if a threshold level of duodenal pH (approximately 2.5) is attained. The extent of depression of gastric secretion is dependent upon the level of pH produced.

13979

Importance of Progesterone to Induction of Sexual Receptivity in Spayed Female Rats.*

FRANK A. BEACH. (Introduced by William Etkin.) With the assistance of Priscilla Rasquin.

From the Department of Animal Behavior, American Museum of Natural History, New York.

Young and his co-workers[1-3] have reported that although estrogen may induce heat behavior in spayed female rats, the administration of progesterone to estrogen-treated animals markedly increases sexual receptivity. Ball[4,5] was unable to confirm these results. She was of the opinion that her failure may have been due to peculiarities in the strain of rats used. In all of the studies listed the behavior of females has been recorded as nonreceptive, slightly receptive or receptive.

In the course of a longer experiment we have collected some data which tend to resolve some of the differences between the findings of Ball and those of Young and his co-workers. Further, our results include complete and sensitive records of different degrees of receptivity in treated females.

Procedure. Virgin female rats were raised in segregation and ovariectomized at 3 or 4 months of age. Hormone injections and mating tests were initiated approximately 3 weeks after castration.

The effects of 3 types of injections upon sexual receptivity were tested. Seven spayed females were injected with 500 R.U. of estrogen,† and tested for sexual receptivity 66-68 hr later. Eight days after the first estrogen treatment the same 7 animals were injected with 500 R.U. of estrogen followed 48 hr later by 0.5 mg of progesterone.‡ Sex tests were conducted 16-18 hr after the progesterone treatment. The second injection schedule was repeated 3 times at 2-week intervals, and mating tests were carried out in each instance.

Six spayed females were injected with 100 R.U. of estrogen followed 48 hr later by 0.5 mg of progesterone. Sex tests were conducted 16-18 hr after the progesterone injection. Two weeks later these same 6 females were injected with 500 R.U. of estrogen followed 48 hr later by 1 mg of progesterone, and mating tests occurred 16-18 hr after the injection of progesterone. After a 2-week interval the second injection schedule was repeated, and sex tests were given again.

Fourteen spayed females received 500 R.U. of estrogen followed 48 hr later by 0.5 mg of progesterone and were tested for receptivity 16-18 hr after the progesterone treatment. Members of this group were given 3 sex tests at 2-week intervals, each test being preceded by the hormone treatment described above.

In the conduction of sex tests the female was placed with a sexually-receptive male in a circular metal cage 30″ in diameter. During the 5-min observation period the following items were recorded: times the male mounted the female, times the female displayed lordosis, frequency of the hopping reaction, of the ear-wiggling reaction and of the back-kicking response of the female. Rapid head movements which produce vibration of the ears, abrupt hopping movements,

* This study was supported by a grant from the Committee for Research on Problems of Sex, National Research Council.

1 Boling, J. L., Young, W. C., and Dempsey, E. W., *Endocrinology*, 1938, **23**, 182.

2 Boling, J. L., and Blandau, R. J., *Endocrinology*, 1939, **25**, 359.

3 Boling, J. L., Blandau, R. J., Rundlett, B., and Young, W. C., *Anat. Rec.*, 1941, **80**, 155.

4 Ball, J., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 416.

5 Ball, J., *J. Comp. Psychol.*, 1939, **28**, 273.

† Estradiol benzoate (Progynon-B) was generously supplied by the Schering Corp., Bloomfield, N.J.

‡ Ciba Pharmaceutical Products, Inc., Summit, N.J., kindly provided the Lutocylin employed.

TABLE I.
Comparison of Sexual Behavior Produced in Spayed Female Rats by the Various Amounts of Estrogen and Progesterone.

Hormone dosage	No. rats	Tests per rat	Average per rat per test: Times mounted	Lordosis responses	Ear wiggling responses	Hopping	Back-kick
500 R.U. estrogen	7	1	17.4	2.4	0.3	0	4.0
500 R.U. estrogen + 0.5 mg of progesterone	7	3	13.4	10.8	5.5	8.4	0.8
100 R.U. estrogen + 0.5 mg of progesterone	6	1	10.3	3.5	0.2	0.7	3.3
500 R.U. estrogen + 1 mg of progesterone	6	2	7.4	6.2	1.9	2.3	2.3

and the exhibition of lordosis in response to mounting by a second animal are characteristic items in the estrus behavior of the normal female rat. Back-kicking is a form of resistance against the male's copulatory attempts.

Results. Data presented in Table I show plainly that rats of our experimental strain are only slightly responsive to estrogen. Two of 7 animals exhibited some indication of receptivity following estrogen injection. For the group as a whole only 14% of the male's mounts elicited lordosis. The tendency to resist the male by back-kicking was strong. The administration of estrogen followed by progesterone resulted in marked increase in sexually receptive behavior in all cases. For the entire group 74% of the male's mounts elicited lordosis. Ear wiggling and hopping behavior were greatly increased, and back-kicking was reduced. Two females which had shown some receptivity under the influence of estrogen alone displayed a much higher degree of behavioral estrus when progesterone was employed.

The administration of 100 R.U. of estrogen followed by 0.5 mg of progesterone induced heat behavior in 5 of 6 rats. Data shown in Table I reveal that when these same animals were given 500 R.U. of estrogen followed by 1 mg of progesterone sexual receptivity was increased and resistance to the male decreased. Under the influence of the first dosage employed the females showed lordosis in response to 34% of the male's mounts. When the larger amounts were administered 84% of the male's mounts elicited lordosis.

The 14 females receiving 500 R.U. of estrogen and 0.5 mg of progesterone were mounted a total of 465 times in 3 tests. Lordosis occurred in 389 instances. In this case 83% of the male's mounts elicited lordosis. The scores on hopping and ear wiggling and back-kicking were closely comparable to those exhibited by the 7 females whose responses to the administration of 500 R.U. of estrogen followed by 0.5 mg of progesterone are shown in Table I.

Discussion. Results reported above indicate that in spayed females from our rat colony sexual receptivity depends upon the synergistic action of estrogen and progesterone. At this point it may be stated that normal estrus females from this colony tested with the same technics herein employed usually exhibit lordosis in response to 80% to 90% of the male's mounts. Individual differences are marked and there is some variation for the same individual in different cycles. However, it is safe to state that degrees of receptivity induced in spayed animals by 500 R.U. of estrogen and 0.5 mg of progesterone are closely comparable to those observed in normal estrus females from the same stock. Our findings are therefore comparable to those reported by Young and his co-workers, and dissimilar to the observations described by Ball.

Our colony was originally derived from Wistar stock, but in the past 12 years wild males have been used as breeders at approximately 4-year intervals. In the intervening periods the colony has been inbred. We are inclined to agree with Ball in her conclusion that animals with which she worked are somewhat unique, and we regard it as prob-

able that in the majority of laboratory strains a high degree of receptivity depends upon the administration of estrogen and progesterone.

The method of counting the male's mounts, the females' lordosis responses, and the frequency of other estrus reactions is to be strongly recommended in all studies of heat behavior. Scores thus obtained provide the basis for a direct quantitative comparison of the effects of various types of hormone treatment. By employing the ratio of lordosis responses to mounts the degree of heat behavior induced by 100 R.U. of estrogen plus 0.5 mg of progesterone may be described as approximately 40% as intense as that which follows the administration of 500 R.U. of estrogen and 1 mg of progesterone. Receptivity induced by 500 R.U. of estrogen alone is only 19% as intense as that resulting from the administration of the same amount of estrogen plus 0.5 mg of progesterone. If these technics are applied to a sufficiently large number of animals results obtained will have high reliability. In the present instance the groups were small and comparisons must be regarded as approximate.

Summary. Three groups of spayed female rats were injected with various amounts of estrogen and of progesterone, and the sexual receptivity thus induced was measured. 500 R.U. of estrogen produced some receptivity in 2 of 7 cases. The same amount of estrogen followed by 0.5 mg of progesterone resulted in a much higher degree of receptivity in all 7 animals. 100 R.U. of estrogen and 0.5 mg of progesterone induced a low degree of receptivity in 6 of 7 cases. 500 R.U. of estrogen and 1 mg of progesterone caused the same animals to exhibit much more intense heat behavior. It is concluded that in the strain of animals studied the production of estrus behavior of normal intensity involves the synergistic action of estrogen and progesterone.

13980

Relation of Anesthetics to Experimental Shock Syndrome Produced by Venous Occlusion.

J. E. BORQUE AND G. KOTALIK. (Introduced by H. O. Haterius.)

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A major difficulty encountered in studies of the factors affecting the onset and course of experimental traumatic or circulatory shock has been that of failure to bring about a fairly constant and readily reproducible syndrome. A partial solution, at least, seems to be offered by the technic reported by Perlow *et al.*[1] who modified the method of regional venous occlusion (Mann)[2] for this purpose. This approach to the problem has been utilized in our laboratory in a considerable number of animals with gratifying results, and certain experimental variables have been studied. The present report deals with the influence on the experimental picture of certain anesthetic agents.

Methods. The technic of venous occlusion as outlined by Perlow *et al.* consisted of ligation, under anesthesia, of the common and the internal iliac veins, followed by injection of a suspension of animal charcoal either into the external iliac or the femoral vein; the latter, in our hands, has seemed preferable in view of easier access. Anesthetics were given at dose levels biologically standardized for each animal (Wiggers);[3] these usually approximated the calculated doses, which were: (1) morphine sulfate, 2%, 1 cc subcutaneously followed by 1.5 g/kg urethane, 50% intravenously; (2) barbital sodium,

[1] Perlow, S., Killian, S. T., Katz, L. N., and Asher, R., *Am. J. Physiol.*, 1941, **134**, 755.

[2] Mann, F. C., *Am. J. Physiol.*, 1918, **47**, 231.

[3] Wiggers, C. J., *Physiol. Rev.*, 1942, **22**, 74.

TABLE I.
Effect of Anesthetic upon Survival Time in Shock Following Venous Occlusion.

Anesthetic	Duration of anesthetic	No. of exp.	Avg survival time and S.D.	Range in survival time	Wt. incr. of occluded leg, avg %	Range, %
Urethane	Throughout exp.	11	5′18″ ± 2′7″	3′20″-10′45″	2.2*	1.0-3.2
Barbital sodium	″ ″	12	4′50″ ± 1′32″	2′40″-7′15″	2.8	1.1-5.5
Pentobarbital sodium	″ ″	10	7′11″ ± 1′40″	3′25″-9′20″	2.8	1.0-4.7
″ ″	During operation	12	8′16″ ± 4′29″	3′45″-16′45″	4.6	2.1-7.9
Ether	″ ″	12	6′10″ ± 3′8″	3′25″-16′	3.6	2.6-5.3

*Weight increase/body weight.

10%, 225 mg/kg intravenously; (3) pentobarbital sodium, 3%, 30 mg/kg intravenously; (4) ether, by mask.

Complete surgical anesthesia was induced in all cases. Moreover, it was maintained throughout the observational periods in the urethane, barbital sodium, and in one series of pentobarbital sodium experiments. Anesthesia during the surgical procedure only (aseptic technic) was employed in one pentobarbital series and in the animals in which ether was used.

In a representative group of experiments the following data were obtained prior to and at intervals following occlusion: Mean arterial blood pressure (by Hg manometer, from the common carotid); respiratory rate and heart rate; rectal temperature; hematocrit; hemoglobin (g %) and specific gravity (falling drop method). Blood samples were taken by femoral puncture.

Body weights ranged from 5 to 20 kg, most of the animals weighing from 7-10 kg.

An approximate value for the loss of fluid into the occluded leg was determined by separating the hind limbs (gluteal muscles included) along midline. Differences in weight are expressed in per cent of body weight.

Results. Number of experiments, average survival times and average weight increases of the occluded limbs are given in Table I.

A progressive acceleration in heart rate occurred from 1-2 hr after occlusion, and persisted until terminal stages. This was paralleled by a steady decline in arterial blood pressure which, however, frequently rose from 10-30 mm above control levels before the decrease began.

Blood studies invariably revealed a greater or less degree of hemoconcentration. Barbital sodium and pentobarbital sodium gave closely corresponding control values (Table II).

Changes in rectal temperature following occlusion were not consistent; in the barbital sodium experiments an increase occurred in 7, decrease in 2 and no change in 1; with pentobarbital sodium 5 showed an increase, 4 a decrease and 1 no change; with urethane, decreases in 4, increase in 2, and no change in 2.

Discussion. The average survival time was longest in the pentobarbital sodium series (Table I) and, as might be expected, the degree of hemoconcentration was most marked (Table II). Statistical analysis of the data failed to demonstrate any significant differences in the survival times. In view of this observation and because of its greater ease of administration, pentobarbital sodium is our choice for routine experiments. Maintenance of anesthesia throughout the experiment or for operation only appeared to have

TABLE II.
Effect of Anesthetics on Hemoconcentration in Venous Occlusion Shock.

Anesthetic	Hematocrit			G % hemoglobin			Specific gravity		
	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)
Urethane	48.7	55.8	9.2	17.6	20.2	14.2	1.0560	1.0631	.62%
Barbital sodium	43.3	51.5	14.5	14.3	16.0	23.0	1.0542	1.0597	.52%
Pentobarbital sodium	43.3	56.3	29.7	15.6	21.9	40.3	1.0588	1.0767	1.2 %

*(a) control, (b) maximum, (c) % increase.

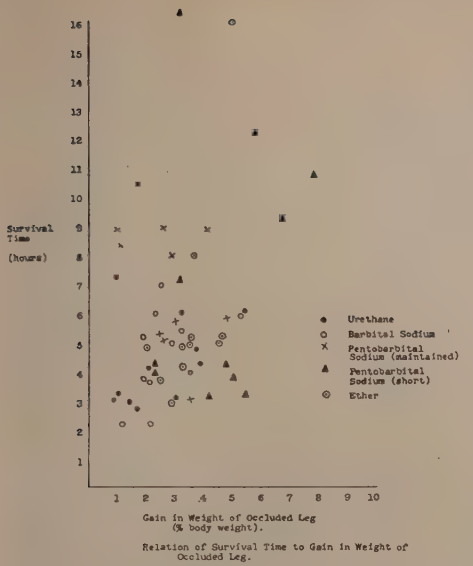

Fig. 1.
Relation of survival time to gain in weight of Occluded Leg.

little influence on the outcome as judged by the results of the 2 pentobarbital series. This circumstance may have been due to 2 factors: the long-lasting effect of the initial injection of pentobarbital sodium and the fact that additional anesthetic was not required when the mean arterial blood pressure had fallen to about 70 mm Hg.

The wide variation in the values for the loss of fluid into the occluded leg (1.0-7.9%) indicates that although reduced blood volume may be a contributing factor, it is probably not the only factor in this type of experimental shock. This is also supported by the lack of correlation between length of survival time and weight of the occluded leg (Fig. 1). The closest approach to a correlation is perhaps suggested in the ether series.

Summary. In this laboratory the method of venous occlusion with several types of anesthetics has consistently produced experimental shock characterized by hemoconcentration, circulatory failure and death. Although oligemia may be a contributing factor, it does not appear to be the only factor in this type of experimental shock. From the point of view of survival time there is little choice between short and maintained anesthesia. Using the same criterion, the anesthetic agents of preference are pentobarbital sodium, and ether.

13981 P

Crystalline Trypsin-Inhibitor and Blood-Clotting.

John H. Ferguson.
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Through the courtesy of Dr. T. E. Weichselbaum, Washington University, St. Louis, we were supplied with 10 mg of pure *crystalline* trypsin-inhibitor (1 mg $\equiv 4.0 \times 10^{-2}$ $[T.U.]_{Hb}$), a polypeptide which was originally isolated from pancreas by Northrop and Kunitz.[1] The sample, dissolved in 10 cc physiological saline, was brought from pH = 5.1 to pH = 7.2 with a trace of n/10 NaOH (= inhib.). The other clotting reagents and test methods have been described in previous publications,[2] the dialyzed crude albumin (= alb.) being prepared from *rabbit* plasma.

I. *Inhibition of clotting of recalcified citrated plasma (rabbit):* Whole plasma and several saline dilutions showed unequivocal retardation of clotting on recalcification in the presence of inhib., as compared to controls; *e.g.*, clotting-time of plasma (1:2 dilution) on adding $CaCl_2$: (a) control (with saline) = 3′ 20″; (b) with inhib. = 7′ 05″.

[1] Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, 1936, **19**, 991.

[2] Ferguson, J. H., *J. Lab. and Clin. Med.*, 1938, **24**, 273.

TABLE I.

Effect of Crystalline Trypsin-Inhibitor (inhib.) and Crude Plasma Albumin (alb.) on Conversion of Prothrombin to Thrombin. Temp. = 21°C, pH = 7.2. Clotting-times, in Seconds, for 0.5 cc Thrombic Mixture (Prothrombin + Ca + Thromboplastin + Inhibitor) *plus* 1.0 cc Fibrinogen.

		Incubation period				
Exp.	"Inhibitor"	2½ min	5 min	10 min	20 min	30 min.
1 a.	none (control)	—	200″	70″	42″	35″*
1 b.	alb.	—	129″	36″	22″	21″
1 c.	alb. + inhib.	—	147″	62″	44″	45″
2 a.	none (control)	25″	16″	16″	—	—
2 d.	inhib.	38″	22″	22″	—	—

* Optimum (checked at 60′ and 90′)

II. *Inhibition of thrombic clotting of fibrinogen:* A 1:100 dilution of Parfentjev's[3] "rabbit clotting globulin" (Lederle Lab.) proved an excellent and stable thrombin (T). The following clotting-times (C.T.) were determined at room temperature (21°C) and pH = 7.2, the thrombin, in each case, being mixed with the cited agents for 10 min prior to the addition of the fibrinogen (F):

1. 1 cc F + 0.5 cc T + 0.5 cc saline .....C.T. = 23 sec.
2. 1 cc F + 0.5 cc T + 0.25 cc saline + 0.25 cc inhib.....C.T. = 24 sec.
3. 1 cc F + 0.5 cc T + 0.25 cc saline + 0.25 cc alb.....C.T. = 48 sec.
4. 1 cc F + 0.5 cc T + 0.25 cc inhib. + 0.25 cc alb.....C.T. = 2760 sec.

No. 4 shows a definite antithrombic effect, as compared with the absence of any direct action of the inhibitor, alone, on the thrombin-fibrinogen interaction (No. 2).

III. *Effect of inhibitor on thrombin formation:* In the routine method[2] for quantitating the activation of prothrombin to thrombin, 0.5 cc of thrombic mixture is sampled after the cited incubation periods and clotting-time noted on adding sample to 1.0 cc test fibrinogen. Thrombic mixtures have a uniform 5 cc volume, made up with saline, as necessary. Each contains 4 cc of Howell-type prothrombin *plus* "activators" and any inhibitors it is desired to study. Two separate experiments are included in Table I, each control (a) employing, as "activators," 0.25 cc N/10 $CaCl_2$ + 0.25 cc dil. brain thromboplastin. In addition, the inhibitory tests have (b) 0.25 cc alb., (c) 0.25 cc alb. + 0.25 cc inhib. or (d) 0.25 cc inhib., respectively. The optimum (shortest) clotting-time is a relative measure of thrombin yield, for each particular experiment.

The few seconds prolongation of optimal clotting-time in the presence of inhib. is significant (*cf.* II) and resembles data previously reported[4] for heparin, except that it is very weak. There is no evidence that the crude albumin supplies a first-phase cofactor, but no significant conclusions as to the possible extent of first-phase inhibitions by the crystalline polypeptide could be reached for at least three reasons, viz. 1. insufficiency of inhib., 2. interfering effect of the incidental (previously noted[4]) thromboplastic effect of the crude albumin (which is probably associated with the presence of serum-tryptase), 3. considerations regarding a "time-factor" (v. infra).

IV. *Antifibrinolytic effect:* The use of thymol-saline kept the tubes free from bacterial growth and it was noted that, whereas the controls (with saline) showed "spontaneous" fibrinolysis complete on the 3rd day, such fibrinolysis was incomplete until the 4th day in the tubes containing albumin (alone) and entirely absent, even at the end of a week, in the tubes containing crystalline inhibitor (with or without alb.). This confirms our previously expressed view that the natural "fibrinolysin," so frequently present, in very small traces, in fibrinogen and other plasma protein products, is none other than the same *serum-tryptase* to which we ascribe the important thromboplastic and variable

[3] Parfentjev, I. A., *Am. J. Med. Sci.*, 1941, **202**, 578.

[4] Ferguson, J. H., *Am. J. Physiol.*, 1941, **134**, 47.

incidental (*e.g.* thrombinolytic or "progressive" antithrombic) effects.

Summary. The data, as a whole, clearly indicate that the crystalline polypeptide tested has a weak inhibitory action on blood-clotting systems. There is good evidence for an antifibrinolytic as well as for antiprothrombic and antithrombic effects. A co-factor seems to be necessary, at least for the last, and it can be supplied in crude plasma albumin. The similarities to the actions of (small quantities of) heparin[4] are striking and the minor discrepancies may be due to such experimental variables as impurity of the albumin, inadequate amounts of inhibitor, and other experimental conditions. In contrast to the "immediate" character of typical heparin effects,[4] there is, in the present experiments, a definite suggestion of a *time-factor* required for the full development of the antithrombic and other inhibitory effects. This may very well explain part of the difficulty encountered in demonstrating more convincingly the first-phase inhibition by the crystalline polypeptide.

These experiments are preliminary and require confirmation with larger amounts of polypeptide and extended study of the question as to optimal conditions for the inhibitory effects. Nevertheless, they are highly suggestive and fit in with a new blood-clotting theory,[5] which revolves around the dominant role of serum-tryptase and its inhibitors in natural blood-coagulation systems.

[5] Ferguson, J. H., *Science*, in press.

13982

A Simple Method for Determining Effective Renal Blood Flow and Tubular Excretory Mass in Man.*

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Quantitative methods for the determination of the tubular excretory mass and the rate of blood flow through the active renal tissue by diodrast clearance have been developed by Smith and his associates[1] and have been used in recent years almost entirely for the purpose of research. We have suggested recently[2-4] that the determination of the effective renal blood flow in patients with arterial hypertension might furnish not only interesting data on the pathogenesis of the disease, but also information of considerable value for the prognosis and selection of patients to be submitted to surgical treatment.

To confirm this possibility it became necessary to find a simple method suitable for the routine clinical study of a large number of patients. The methods of Smith and his associates are not practical as they require continuous intravenous infusion, inlying catheter, the full attention of a physician and a technician for several hours, and a large number of chemical determinations.

To our knowledge, Findley and White[5] made the only attempt to simplify the determination of effective renal blood flow, by the use of a single subcutaneous injection of diodrast. They measured effective renal

* This work was made possible by a grant from the Aaron Mendelson Memorial Trust Fund.

† Research Fellow.

[1] Smith, H. W., Goldring, W., and Chasis, H., *J. Clin. Invest.*, 1938, **17**, 263.

[2] Foà, P. P., Woods, W. W., Peet, M. M., and Foà, N. L., *Arch. Int. Med.*, 1942, **69**, 822.

[3] Foà, P. P., Woods, W. W., Peet, M. M., and Foà, N. L., *Arch. Int. Med.*, in press.

[4] Foà, M. M., Woods, W. W., Peet, M. M., and Foà, N. L., *Univ. Hosp. Bull.*, Ann Arbor, 1942, **8**, 9.

[5] Findley, T., and White, H. L., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 623.

blood flow in 3 hypertensive subjects by 3 consecutive clearance periods. The determinations were not repeated on different days, or controlled by the determination of the ratio of effective renal blood flow to tubular excretory mass. Normal individuals have not been studied. Since the blood flow in hypertensive patients varies widely from case to case, there is no definite standard to which the low values obtained by Findley and White can be compared. Furthermore, the results may have been influenced by the local anesthetic which they used at the site of injection. After subcutaneous injection of diodrast, the concentration of this substance in the plasma is a result, not only of its excretion by the kidney, but also of its absorption from the subcutaneous tissue. For this reason, the concentration of diodrast iodine in the plasma may not remain constant or (it may not) decrease at a uniform rate, and it becomes necessary to determine it in several samples of blood taken during the clearance period.

The following procedure has given very satisfactory results and avoids the disadvantages of continuous intravenous infusion, catheterization, and collection of a large number of specimens. The experiment requires about the same length of time as a urea clearance test.

Procedure. a. Determination of effective renal blood flow: During the hour preceding the experiment, the patient drinks approximately 2000 cc of water to promote diuresis. The test begins with the collection of a specimen of urine, U_0, and of 30 cc of blood, B_0, from the cubital vein. U_0 and B_0 are used as blanks in the chemical determinations. 5 cc of 35% diodrast are then injected intravenously. After 15 min, the patient is asked to empty his bladder completely, the time is exactly recorded and the urine discarded. Ten min later, 30 cc of blood (B_1) are collected and the time recorded. A second sample of blood (B_2) is taken 10 min after B_1 and finally the urine is accurately collected 10 min later. This specimen of urine is usually between 200 and 500 cc and, in these conditions, a normal bladder can be emptied satisfactorily without catheterization. When greater accuracy is desired, especially if the flow of urine is below 8-10 cc/min or if the patient cannot urinate freely, it is advisable to use a catheter and to wash the bladder once or twice with 20 cc of saline.

b. Determination of tubular excretory mass: The patient drinks another 500 cc of water and about 30 min later, 30 cc of 35% diodrast are slowly injected intravenously. Within a minute or two after the injection, the bladder is emptied with care and the urine discarded. Approximately 5 and 15 min thereafter, specimens of blood (B_3 and B_4) are taken from the cubital vein and about 5 min after the collection of B_4, the urine is accurately collected.

This schedule can be modified, but it is necessary that the time of each collection of blood and urine be recorded exactly. When the patient is sufficiently coöperative and when each collection of urine can be timed at will, only one sample of blood need be taken during each urine collection period—this blood must be collected *exactly* at the *mid-point* of the urine period.

The diodrast is determined as iodine following the method of Alpert.[6] The computation of the diodrast clearance is based on the usual formula: $C_D = UV/P$, where C_D = diodrast clearance; U = concentration of diodrast iodine in the urine; V = volume of urine in cc per minute; and P = concentration of diodrast iodine in the plasma at the mid-point of the urine collection. If only one sample of blood is taken at the mid-point, P is equal to the concentration of diodrast iodine in this sample. If 2 samples of blood are taken, the concentration of iodine in the plasma is plotted against time and the desired concentration read on the curve at the time corresponding to the mid-point of the period of urine collection. The diodrast plasma clearance is equal to the effective renal plasma flow. A hematocrit determination permits the conversion of this value to blood flow. The tubular excretory mass is computed according to Smith *et al.*, again from the concentration of diodrast iodine in the blood at the mid-point of urine collection. The filtration rate, necessary for

[6] Alpert, L. K., *Bull. Johns Hopkins Hosp.*, 1941, **68**, 522.

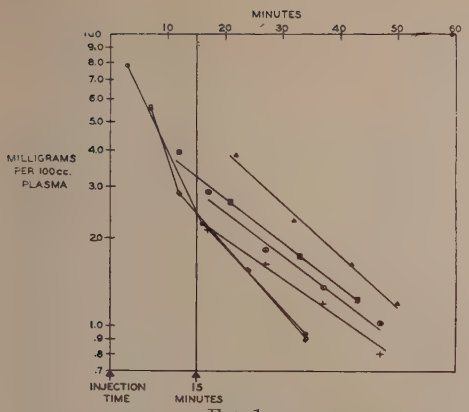

FIG. 1.
Concentration of Iodine in the Plasma After Intravenous Injection of 5 cc of Diodrast.

the computation of Diodrast Tm, is obtained by simultaneous determination of the inulin clearance by the method of Alving and Miller.[7]

As shown in Fig. 1, the concentration of diodrast iodine in the plasma, following a single intravenous injection of 5 cc of 35% diodrast, falls sharply for about 15 min. This period probably corresponds to the time necessary for the diodrast to diffuse into the extracellular fluid. Following this period of equilibration the concentration of iodine in the plasma decreases slowly and in a straight line from 2-4 mg to 0.7-1.5 mg per 100 cc of plasma in about 30 min. The concentration at the mid-point of the urine collection period therefore represents the mean concentration of iodine during the whole period and can be used for the computation of the diodrast clearance for that period. The diodrast clearance, at these concentrations of diodrast in the plasma, is equal to the effective plasma flow.

For the determination of tubular excretory mass, the concentration of diodrast iodine should be maintained above approximately 15 mg of iodine per 100 cc of plasma. This is obtained by a single intravenous injection of 30 cc of 35% diodrast. Following this injection, the concentration of iodine in the plasma falls from approximately 23-40 mg to approximately 14-18 mg per 100 cc of plasma, remaining sufficiently high throughout the whole clearance period to allow the

[7] Alving, A. S., and Miller, B. F., *Arch. Int. Med.*, 1940, **66**, 306.

TABLE I.
Effective Renal Blood Flow and Tubular Excretory Mass in Eleven Normal Subjects.

	Subject age, sex	Blood pressure	Diodrast plasma clearance, C_D cc/min	Effective renal blood flow, cc blood/min	Tubular excretory mass Tm_D, mg I_2/min	C_D/Tm_D	Inulin clearance during determination of Tm_D, cc plasma/min
1.	62 M	165/125	424	785	34	12.6	94
		150/94	465	832	31	14.8	169
		*125/90	514	900	38	15.4	53
		170/105	461	842	—	—	—
2.	30 M	110/80	465	786	45	10.1	95
		120/80	513	871	47	10.9	116
3.	26 M	122/68	467	800	—	—	—
		—	548	915	40	13.6	124
4.	29 M	110/75	717	1245	39	18.5	100
5.	30 M	110/60	695	1262	47	14.9	144
6.	37 M	122/81	518	849	25	23.4	68
Avg of 6 males		120/78	566	987	39	15.9	108
7.	23 F	105/65	754	1134	—	—	—
		95/50	694	1061	39	17.6	145
8.	58 F	160/90	534	791	31	17.9	103
		* —	552	817	—	—	—
9.	27 F	105/62	485	794	41	11.9	105
10.	45 F	105/70	440	710	30	14.8	142
11.	40 F	128/80	651	1017	25	26.1	101
Avg of 5 females		119/72	568	885	33	17.6	119
Total avg		120/75	566	940	36	16.7	113

*Under ether anesthesia.

determination of the tubular excretory mass.

If greater accuracy is desired, the calculation should take "delay time" into account.‡ Two samples of blood should be collected during the clearance period and the plasma concentration taken 150 sec before the midpoint of the urine period. This was done in five of our subjects and the difference between the value given in the table and the corrected value averaged 7.78% (5.66 to 9.9%). This difference has very little clinical significance, and becomes negligible when the rate of urine flow is 15-20 cc/min or more.

Results. Effective renal blood flow and tubular excretory mass were determined in 11 normal subjects (6 males and 5 females). In 5 subjects the determinations were repeated after an interval of a few days. As shown in Table I, the values obtained are well within the normal range as determined by Smith's method[1,2] and the determination can be repeated in the same patient with good agreement.

Summary. A simple method for the determination of effective renal blood flow and tubular excretory mass in human subjects is described. Continuous intravenous infusion and catheterization are avoided. The method is suitable for routine clinical use.

‡ for the significance of "delay time" see Smith *et al.*[1]

The diodrast used in this study was generously supplied by the Winthrop Chemical Co.

13983 P

Effect of Testosterone Therapy on Concentration of Potassium in Serum.*

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In his pioneer investigations on perfusion fluids, Ringer observed that while the addition of potassium was not essential for the maintenance of the frog or turtle heart beat, a concentration of approximately 4 milliequivalents per liter was one of the requisites of a solution providing optimal contractions. For the mammalian heart Locke designated the optimal potassium concentration of perfusion fluids at approximately 5.5 meq. per liter.

The observation that potassium salts relieved the paralysis of so-called familial periodic paralysis[1-6] and the more recent observation that a fall in serum potassium concentration was associated with the attacks of paralysis suggested a correlation between muscle weakness and serum potassium concentration.[2-6] During such attacks bradycardia, hypotension, arrhythmia and cardiac dilatation have been observed. However, though a fall in the serum potassium concentration occurs with such attacks, the specific concentration at which paralysis appears varies greatly. A somewhat similar concomitant decrease in serum potassium and in muscle strength has been observed following the administration of desoxycorticosterone acetate[7-13] and the skeletal mus-

* This investigation was aided by a grant from the Commonwealth Fund, New York City.

1 Mitchell, J. K., Flenner, S., and Edsall, D. L., *Trans. Assn. Am. Physicians,* 1901, **16**, 268.

2 Aitken, R. S., Allott, E. N., Castleden, L. I. M., and Walker, M., *Clin. Sc.,* 1937, **3**, 47.

3 Allott, E. N., and McArdle, B., *Clin. Sc.,* 1938, **3**, 230.

4 Ferrebee, J. W., Atchley, D. W., and Loeb, R. F., *J. Clin. Invest.,* 1938, **17**, 504.

5 Gannon, G. D., Austin, J. H., Blithe, W. D., and Reid, C. G., *Am. J. Med. Sc.,* 1939, **197**, 326.

6 Talbott, J. H., *Medicine,* 1941, **20**, 85.

7 Ferrebee, J. W., Ragan, C., Atchley, D. W., and Loeb, R. F., *J. A. M. A.,* 1939, **113**, 1725.

8 Thorn, G. W., Howard, R. P., and Emerson, K., Jr., *J. Clin. Invest.,* 1939, **18**, 449.

TABLE I.
Effect of Testosterone Therapy on the Concentration of Serum Potassium.

Subject	Diagnosis	Date	Serum K Meq. per liter	Daily therapy NaCl g	DOCA* mg	Me-T† mg	Test-P‡ mg
M.M.	Addison's disease ♀	5-11	5.3	9	3	—	—
		6-15	3.8	9	3	—	—
		6-29	2.9	9	3	90	—
		7-6	1.9	9	3	—	25
		7-9	1.9	9	3	—	25
		7-14	3.8	9	3	—	—
		7-17	4.8	9	3	—	—
		7-25	5.6	9	—	—	—
		7-29	2.6	9	—	—	50
		8-10	0.7	9	—	—	50
		8-13	4.7	9	—	—	—
R.Y.	Dwarf ♂	8-21	0.6	—	—	30	—
D.W.	Dwarf ♂	8-12	4.2	—	—	—	—
		9-9	4.1	—	—	—	25
		9-14	0.4	—	—	—	50
		10-16	3.5	—	—	20	—
Le.B.	Hyperthyroid ♀	8-26	5.0	—	—	—	—
		9-9	3.6	—	—	—	25
		9-24	4.2	—	—	50	—
		10-7	1.5	—	—	100	—

*DOCA equals desoxycorticosterone acetate administered I.M. 4-29 to 7-17.

†Me-T equals methyl-testosterone administered orally for patient M.M. from 6-16 to 6-29; for patient R.Y. from 7-17 to 8-21; for patient D.W. from 9-16 to 10-16; for patient Le.B. 50 mg from 9-11 to 9-24; 100 mg from 10-3 to 10-7.

‡Test-P equals testosterone propionate administered I.M. for patient M.M., 25 mg from 6-30 to 7-8; 50 mg from 7-25 to 7-31, 8-4 to 8-10; for D.W. 25 mg from 8-30 to 9-9 and 50 mg from 9-10 to 9-14; and for patient Le.B. 25 mg from 8-30 to 9-10.

cle weakness and the heart failure that may occur with such therapy has been ascribed to these low serum concentrations.[9,11,13,14] On the other hand, low serum potassium levels may occur without paralysis or heart failure following the injection of insulin.[15,16]

This paper reports the striking fall in serum potassium which we occasionally have observed in patients following the daily administration of either methyl testosterone by mouth or testosterone propionate intramuscularly. The analyses were carried out on 4 to 8 cc of non-hemolyzed serum by the method of Fiske and Litarczek.[17] So far as we are aware the serum potassium concentrations presented in the table include the lowest values recorded in medical literature.

The subjects during the periods of these low values were up and about, suffered no muscular weakness or alterations in their electrocardiograms. Detailed metabolic studies of subject M.M., will be reported elsewhere.[18]

From the previous observations referred

[9] Kuhlmann, D., Ragan, C., Ferrebee, J. W., Atchley, D. W., and Loeb, R. F., *Science*, 1939, **90**, 496.

[10] Ragan, C., Ferrebee, J. W., Phyfe, P., Atchley, D. W., and Loeb, R. F., *Am. J. Physiol.*, 1940, **131**, 73.

[11] Talbott, J. H., and Schwab, R. S., *New Eng. J. Med.*, 1940, **222**, 585.

[12] Miller, H. C., and Darrow, D. C., *Am. J. Physiol.*, 1941, **132**, 801.

[13] McGavack, T. H., *J. Clin. Endocrin.*, 1941, **1**, 68.

[14] Darrow, D. C., and Miller, H. C., *J. Clin. Invest.*, 1942, **21**, 601.

[15] Briggs, A. P., Koechig, E. A., Doisy, E. A., and Weber, C. J., *J. Biol. Chem.*, 1923, **58**, 721.

[16] Harrop, G. A., and Benedict, E. M., *J. Biol. Chem.*, 1924, **59**, 683.

[17] Fiske, C., and Litarczek, *Folin's Laboratory Manual of Biological Chemistry*, 5th Edition.

[18] Talbot, N. B., Butler, A. M., and MacLachlan, E., unpublished data.

to above and the data reported here, it would appear that the circumstances associated with the low serum potassium concentrations encountered in clinical medicine may be outlined as follows:

1. A fall in serum potassium with testosterone therapy appears to reflect an increase in total tissue potassium associated with an increase in tissue nitrogen consistent with an increase in tissue mass.[18] It is not associated with weakness or paralysis or marked alterations in blood sugar.

2. A fall in serum potassium and decrease in blood sugar following the injection of insulin appears to be associated with a decreased urinary potassium and phosphorus excretion and an increased intracellular potassium and phosphorus (possibly without increase in muscle mass). It is not associated with loss of muscle contractility.

3. A fall in serum potassium during attacks of periodic paralysis is associated with a decreased urinary excretion and increased tissue retention of potassium and phosphorus similar to that observed following insulin but is not associated with alterations in blood sugar.

4. A fall in serum potassium that follows excessive administration of desoxycorticosterone is associated with no change in blood sugar, an increased urinary excretion of potassium, a decrease in muscle potassium concentration, an increase in muscle sodium concentration, and a decrease in muscle strength.

Finally in experiments on rats[12] it has been shown that within wide limits neither the amounts of potassium in muscle cells nor abnormally low concentrations of potassium in the serum limit the capacity of rats to swim.

Thus our observations on the fall in serum potassium concentration and alterations in intracellular potassium balance during testosterone therapy supplement the existing evidence indicating that such changes in potassium distribution do not *per se* determine muscle contractility.

13984

Comparison of pH and Population of *Trichomonas Foetus*.*

BANNER BILL MORGAN. (Introduced by M. R. Irwin.)

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Witte[1] first showed that *Trichomonas foetus* could tolerate a pH range of 5.5 to 8.5. This was later confirmed by Riedmuller[2] who also reported that the optimal pH for *T. foetus* in pure culture was 6.5 to 7.5. Lyford[3] demonstrated the adaptability of *T. foetus* in pure cultures to various hydrogen-ion concentrations with final pH readings of 4.8 and 5.2. Morisita[4] stated the optimum pH for *T. foetus* ranged from 6.6 to 7.8; the flagellate could survive between 5.6 and 8.4, and the range during maximum growth was 5.4 to 5.6. Johnson[5] reported the population in relation to pH with a pure culture of *T. vaginalis*. Prior to this report, the relationship had not been completely established with *T. foetus*.

This paper is concerned with the correlation between pH and population of several pure culture strains and 2 strains of *T. foetus* in association with an atypical strain of *Corynebacterium renalis*.† This strain is unable to ferment dextrose.

Strain A,‡ originally isolated by Glaser and

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Project No. 622-V; Trichomoniasis and other reproductive diseases of cattle.

1 Witte, J., *Zentr. f. Bakt.*, 1933, **128**, 188.

2 Riedmuller, L., *Ibid.*, 1936, **137**, 428.

3 Lyford, H., *Am. J. Hyg.*, 1941, **33**, 69.

4 Morisita, T., *Jap. J. Exp. Med.*, 1939, **17**, 1.

5 Johnson, G., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 567.

Coria,[6] strains B, and C, isolated by the writer,[7] and strain D,§ were used in this work. The culture medium used was a modification of Schneider's[8] citrate; whole egg and defibrinated bovine blood slants overlaid with 5% bovine serum in modified citrate solution, sterilized by autoclaving at 15 pounds pressure for 30 min. The pH of the fluid medium was adjusted with N/1 HCl or N/1 NaOH. The incubation temperature was 37 ± 1°C. The pH determinations were made with a Coleman 200 Electrometer which was checked against a standard buffer.

The flagellates were counted in a Neubauer hemacytometer every 12-24 hr until motility of the organisms ceased. Twenty cultures were examined at each pH. Each tube was inoculated with 100,000 organisms from a 96-hr culture of the various strains mentioned above. The pH of the original medium was set at 7.2. Total amount of fluid in each tube was set at 5 cc. Controls consisted of uninoculated tubes. Blood agar plates were used to check bacterial contamination.‖ (Veal infusion agar + 5% bovine blood).

Strain A. Maximum numbers occurred between pH 6.1 and 6.3 at 96 hr with a count of approximately 3 million organisms per cc. Final pH was 5.4. (Fig. 1).

Strain B. Maximum numbers occurred between pH 5.4 and 5.8 at 96 hr with a count of approximately 4.5 million organisms per cc. Final pH was 5.2. (Fig. 2).

Strain C. Maximum numbers occurred between pH 5.7 and 5.9 at 96 hr with a count of 4 million organisms per cc. Final pH was 5.3. (Fig. 4).

Strain C + Corynebacterium renalis.

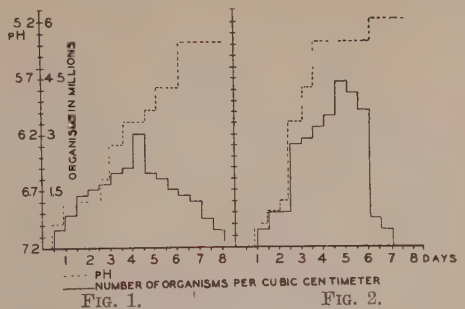

FIG. 1. FIG. 2.

Figs. 1 and 2 show the maximum numbers and pH of strains A and B, respectively.

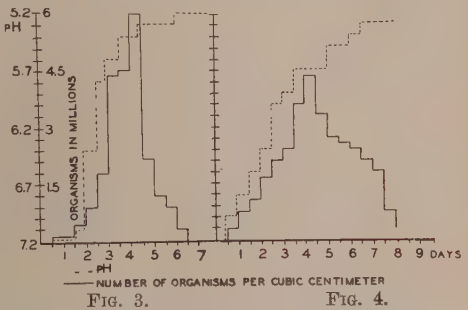

FIG. 3. FIG. 4.

Figs. 3 and 4 show the maximum numbers and pH of strain C + *Corynebacterium renalis* and in bacteria free-cultures, respectively.

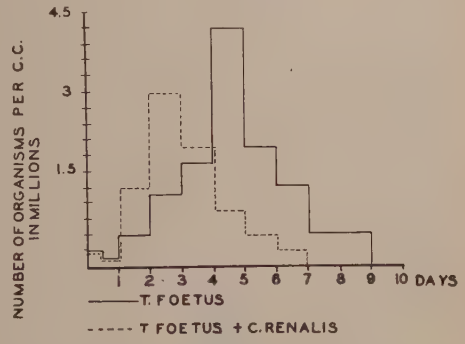

FIG. 5.

Fig. 5 shows the maximum numbers and pH of strain D in bacteria-free cultures and in the presence of *Corynebacterium renalis.*

† The writer wishes to thank Dr. I. A. Merchant and Dr. S. Kenzy, Department of Veterinary Hygiene, Iowa State College, and Miss M. Bernstein, Department of Veterinary Science, University of Wisconsin, for identification of this strain of bacteria.

‡ Strain A was supplied by Dr. A. Tatum and Dr. J. Byrne, Department of Pharmacology, University of Wisconsin.

6 Glaser, R., and Coria, N., *Am. J. Hyg.*, 1935, **22**, 221.

7 Morgan, B. B., and Wisnicky, W., *J. Am. Vet. Med. Assn.*, 1942, **100**, 783.

§ Strain D was supplied by Dr. B. Schwartz, Zoological Division, U.S.D.A., Washington, D.C.

8 Schneider, M., unpublished thesis, Library, Univ. Wis., 1941.

‖ The writer wishes to thank Miss Myrtle Bernstein, Department of Veterinary Science, University of Wisconsin, for making the bacterial examinations.

Maximum numbers occurred between 5.3 and 5.6 at 72 hr with a count of 6 million organisms per cc. Final pH was 5.2. (Fig. 3).

Strain D. This strain was received in a contaminated state with an atypical strain of *C. renalis*. It was isolated bacteria-free by a modification of the capillary migration of Stone and Reynolds.[9] The pH of strain D was essentially the same as that of Strain A. Counts were made every 24 hr. Maximum numbers occurred between pH 6.1 and 6.3 at 96 hr with a count of approximately 4 million organisms per cc. Final pH was 5.4.

Strain D + Corynebacterium renalis. Maximum numbers occurred between pH 5.7 and 5.9 at 48 hr with a count of approximately 3 million organisms per cc. (Fig. 5). Two sets of controls were run, one set being uninoculated, the other with *Corynebacterium renalis*. Both sets of controls changed their pH from 7.2 to 6.8. This bacterium apparently grows in close association with *T. foetus* as the flagellates multiply more rapidly in its presence than in bacteria-free cultures. This acceleration is difficult to explain. There may be a change in the medium due to the hydrolyses of proteins and carbohydrates by the bacterium, but the nature of this is not known.

9 Stone, W., and Reynolds, F., *Science*, 1939, **90**, 91.

13985

Impedance Changes Induced in the Brain by Electric Stimulation.

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The extensive application of electric stimulation of the brain, not only experimentally but also for therapeutic purposes as in the so-called electric shock treatment of certain psychoses, makes it desirable to get a more intimate knowledge of the effects of electric stimuli and the subsequent excitation upon the state of the cells of the central nervous system. It is known that excitation of peripheral nerves is associated with decrease in D.C. resistance (Hermann,[1] Ebbecke[2]) and A.C. impedance (Lullies,[3] Cole and Curtis[4]) respectively. Since such changes may be able to throw some light upon the state of the cellular surface films (Gildemeister),[5] it seemed of interest to ascertain whether similar effects are demonstrable in the central nervous system.

For the measurement of the impedance a Wheatstone bridge arrangement energized with A.C. of from 500 to 6000 cycles was used as previously described. (Spiegel and Spiegel-Adolf).[6] Quick determinations of the balance were facilitated by a 5-stage amplifier connected not only to a telephone receiver but also to an "electric eye" so that the minimum could be detected by visual as well as by acoustic observations.

In the first series of experiments on cats under superficial ether anesthesia the electrodes were platinum wires coated with platinum black, inserted into the brain substance as in previous experiments (Spiegel and Spiegel-Adolf)[6] or platinum-Ringer electrodes placed with the smallest possible

1 Hermann, L., *Pflueger's Arch. f. d. ges. Physiol.*, 1872, **6**, 560; 1873, **7**, 349.

2 Ebbecke, U., *Pflueger's Arch. ges. Physiol.*, 1922, **195**, 555.

3 Lullies, H., *Pflueger's Arch. ges. Physiol.*, 1930, **225**, 69, 87.

4 Cole, K. S., and Curtis, H. J., *J. Gen. Physiol.*, 1939, **22**, 649.

5 Gildemeister, M., in *Handb. d. norm. u. pathol. Physiol.*, edit, by Bethe, 1928, **8**, II, 657.

6 Spiegel, E., and Spiegel-Adolf, M., *Am. J. Psychiat.*, 1936, **92**, 1145; *J. Nerv. and Ment. Dis.*, 1939, **90**, 188.

pressure on the surface of the brain. The stimulating electrodes were placed on the motor cortex and the testing electrodes (connected to the Wheatstone bridge) on the parieto-occipital region of the same or of the opposite hemisphere.

The convulsions following faradic stimulation of one minute duration were associated with a decrease of impedance, which was more marked if the impedance was determined with a low frequency current (547 cycles per sec.) than on measurement with a current of 5120 cycles per sec. In 10 measurements at the lower frequency the decrease of impedance varied between 0 and 16% (mean value 7.3%) while in 9 measurements at the higher frequency it varied between 0 and 7.2% (mean value 3.4%). This is in agreement with former experiments on metrazol and insulin convulsions (Spiegel and Spiegel-Adolf).[7] Since the convulsive state is associated with changes in brain circulation, which may affect these measurements, it seemed desirable to use an object in which the disturbing influence of circulatory changes could be eliminated.

A second series of experiments was, therefore, performed on surviving frog's brains. Such preparations show spontaneous discharges for as long as 3 hr after removal if kept in a Ringer's solution, as has already been demonstrated by Gerard and Libet;[8] electrical stimulation is able to change the functional state of such surviving brains as is demonstrated by the fact that their potential discharges may be altered by electrical stimulation.

The brain is held between two vertical platinum-Ringer electrodes in a glass chamber that can be filled with Ringer's solution in the intervals between the measurements (Fig. 1). In one method the stimulating electrodes are placed on the frontal ends of the hemispheres (or on the olfactory lobes). In another method the platinum-Ringer electrodes (e_1, e_2) are used for application of the stimulating current as well as for measurement of the resistance. In the latter method the brain is connected alternately to the stimulating and the bridge circuit by means of a double pole double throw switch.

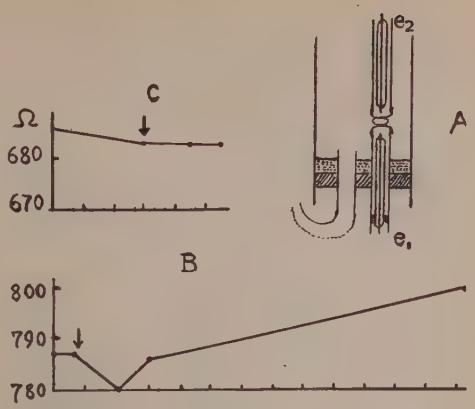

FIG. 1.

1A. Apparatus for experiments on isolated frogs' brains. e_1, e_2—Platinum-Ringer electrodes. Other details described in the text.

1B. Surviving isolated frog's brain. Changes in impedance following application of 10 make and break shocks (at ↓). Abscissa: time in minutes.

1C. Control experiment on brain killed in chloroform. Application of 10 make and break shocks at ↓.

In these experiments also stimulation of the brain was followed by a definite decrease of impedance. This was particularly evident, if there was a spontaneous tendency for the impedance of the resting brain to gradually increase so that the changes following the stimulation were in the opposite direction. In order to differentiate the physiological effect of the stimulation from purely physi-

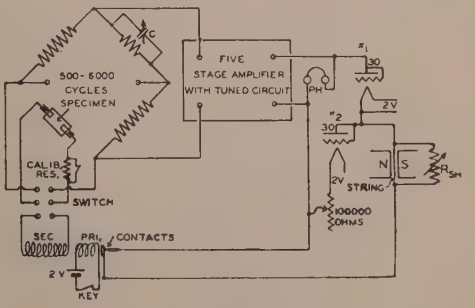

FIG. 2.

Circuit for recording of impedance changes. Details explained in text.

[7] Spiegel, E., and Spiegel-Adolf, M., *J. Nerv. and Ment. Dis.*, 1941, **93**, 750.

[8] Gerard, R. W., and Libet, B., *J. Neurophysiol.*, 1939, **2**, 153.

cal effects (such as heating, polarization), the experiments were repeated after the brain had been killed in chloroform. This was particularly important since measurements with thermocouples showed that, for instance, application of faradic (tetanizing) current through the Pt-Ringer electrodes for one minute at a coil distance of 4 cm increased the temperature on the surface of the frog's brain by from 1.0°C to 1.68°C, which change of temperature was of course by itself able to reduce the impedance of the brain. Instead of faradization we applied, therefore, single induction shocks, (10 make and break shocks), and with this type of stimulation a definite difference between the effect upon

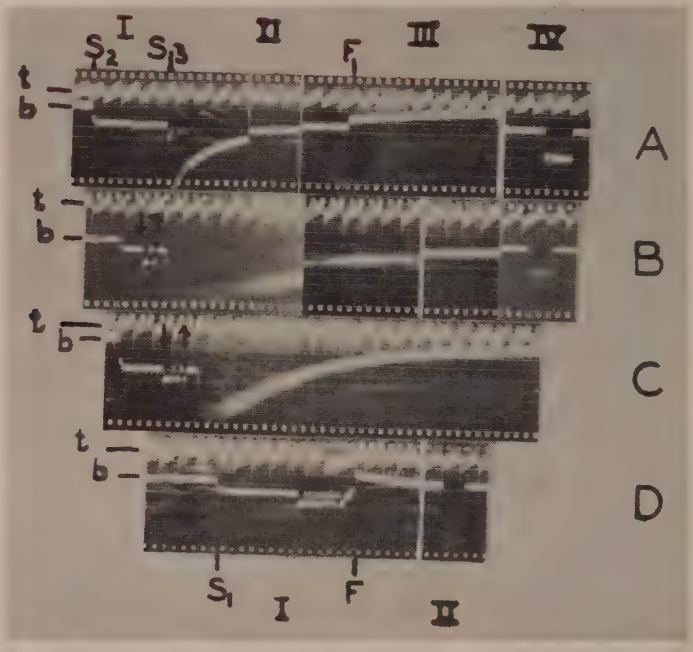

FIG. 3.

Stimulation of Isolated Surviving Frogs' Brains.

A. I. Application of 10 make and break shocks with shunt at 10 Ω (S_2); immediately after stimulation, shunt 1000 Ω (S_3).

II. 2 seconds later (shunt 1000 Ω).

III. 4 seconds later (shunt 1000 Ω), then full sensitivity of string (from F).

IV. Standardization with 20 Ω (shunt 1000 Ω).

B. I. Application of one make and break shock (shunt 1 Ω), then full sensitivity.

At ↓ brain disconnected from bridge and connected to stimulating coil, at ↑ brain reconnected to bridge. Application of stimulus between ↓ and ↑.

II. 6 seconds later.

III. 7 seconds later, full sensitivity.

IV. Standardization with 15 Ω.

C. Application of 2 make and break shocks (shunt 1 Ω), then full sensitivity. ↓ and ↑ as in B.

D. Control experiment on brain killed in chloroform.

I. Application of 10 make and break shocks, shunt 1 Ω (S_1), then full sensitivity of the string (from F).

II. Standardization with 15 Ω (full sensitivity).

t = time in seconds; b = balance position.

the surviving and that upon the dead brain could be demonstrated. While on the latter no or only minimal changes of impedance in either direction could be detected, there was a definite decrease on the surviving brain. (Fig. 1).

It soon became apparent that the changes of conductance took place rather quickly, and that the beginning of this change escaped the measurement, even if special measures were taken to shorten the balancing of the bridge (*e.g.*, setting, during the stimulation, the variable resistance and capacitance at the approximate expected values). The changes of impedance were, therefore, recorded by a string galvanometer in the following way. (Fig. 2).

The zero points of the bridge were connected to a 5-stage amplifier (with a filter circuit tuned to the frequency of the bridge current). The output of the amplifier was connected to head phones to facilitate preliminary bridge balancing and also through a vacuum tube rectifier, with zero balancing circuit, to a string galvanometer. First the balance position of the bridge and its eventual shift were recorded. Then a one or 10 ohm shunt was applied to the string galvanometer to greatly reduce its sensitivity. (Fig. 3). The brain was disconnected from the bridge and connected to the stimulating coil by means of a double pole double throw switch, and make and break shocks were applied. After the stimulation was completed, the specimen was reconnected to the bridge as quickly as possible, and the sensitivity of the string was restored. Thus changes in the impedance of the brain were recorded 1-2 sec following the stimulation, and the gradual return to the previous value could be followed. After each stimulation experiment the circuit was standardized; the bridge was first balanced while a certain additional resistance (*e.g.*, 15Ω) was in series with the brain. One first recorded this balance position of the string and then its deflection after the additional resistance had been removed.

After a single make and break shock the deviation was 1.2-2.5% of the initial value (*e.g.*, deviation 18-37Ω, initial value 1460-1480Ω), and the string returned to the original position after 14-27 sec. (Fig. 3B). After 10 make and break shocks in various preparations changes from 3-10% and a return to the initial value in 9-38 sec were observed. (Fig. 3A). Repetition of the same procedure in control experiments on brains killed in chloroform gave practically no changes in the balance position of the string. (Fig. 3C). Since the string was deflected in the same direction whether the impedance of the specimen increased or decreased from the balance position, it was necessary to establish in which direction the impedance changes actually occurred. This was repeatedly done with the bridge and phones as detector, and it was found that we dealt with a decrease of impedance after stimulation. Studies of the spinal fluid are in progress (with Drs. M. Spiegel-Adolf and E. W. Ashkenaz) which seem to indicate that these changes of impedance are at least partly due to impairment of the brain cells.

Summary. The convulsions following faradic stimulation of the brain are associated with decrease of its impedance (experiments on cats under superficial ether anesthesia). A similar change could also be demonstrated following single induction shocks applied to isolated, surviving frog's brains, where the disturbing influence of the circulation was eliminated, while such a change failed to appear in control experiments on dead brains.

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Resistance of *Staphylococcus aureus* to the Action of Penicillin.*

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During the course of a study of the effect of penicillin therapy in human infections it was found that a favorable response was not always obtained in those patients with infections caused by *Staphylococcus aureus*. The present study was undertaken to determine some of the factors involved in the resistance of *Staphylococcus aureus* to the action of penicillin.

Methods. The sodium salt of penicillin† was dissolved in 0.85% sodium chloride so that the final concentration was 20 Florey units per cubic centimeter. This standard solution of penicillin was used in all *in vitro* tests. In patients with infections the penicillin was administered in a similar solution but the concentration was increased to 1000 Florey units per cc.

The strains of *Staphylococcus aureus* used in this study were all isolated from patients with clinical infections and were stored on blood agar slants until needed.

The method used to determine the sensitivity of staphylococci to the action of penicillin is a modification of a test previously described.[1] In brief, in a series of 11 tubes serial dilutions of 0.2 cc of the standard solution of penicillin was made with 0.2 cc of veal infusion broth. To each tube in the series 0.5 cc of the proper dilution of a 12-hr culture of the test organism was added. Pour plates of each inoculum were made and the number of organisms was found to vary from 1000 to 30,000 per 0.5 cc. The cultures containing penicillin were then incubated at 37°C for 18-24 hr, following which they were examined for turbidity. Subcultures on blood agar plates were made from those tubes near the end point.

Resistant strains were developed *in vitro* by growing the test organism in gradually increasing concentrations of penicillin. A series of 11 cultures tubes containing decreasing amounts of penicillin were set up as described above. The test culture containing from 1000 to 100,000 organisms was added in 0.5 cc of veal infusion broth. At 24-hr intervals transfers were made from the tube showing full growth and containing the greatest concentration of penicillin. At intervals comparative tests using the parent strain of the test organism were run.

Results. The lowest concentration of penicillin required to kill 1000 to 30,000 organisms of each of 29 strains of *Staphylococcus aureus* was determined. It was found that all strains were killed in concentrations between 0.02 and 0.35 Florey units per cc of broth. Two strains were killed by 0.02, 13 by 0.04, 12 by 0.08, 1 by 0.17 and 1 by 0.35 Florey units per cc. These results indicate, then, that different strains of staphylococci vary only slightly in their resistance to penicillin.

Production of Resistant Strains. The variation in the susceptibility of strains of *Staphylococcus aureus* isolated from 14 patients with active infections could not be correlated with the clinical and bacteriological response to penicillin therapy. It therefore was important to study the possibility of acquired resistance to penicillin following prolonged exposure to the antibacterial substance. *In vitro* it was demonstrated in 2 strains that the *Staphylococcus aureus* may so adapt itself that growth takes place in broth containing relatively large amounts of penicillin. The rapidity with which the staphylococci acquired resistance is shown in Table I. Strain Aurice showed an increased resistance of 64-fold after 54 days' exposure

*This study was supported by a grant from the Johnson Research Foundation, New Brunswick, N.J.

† Penicillin was supplied through the courtesy of Dr. George A. Harrop, Squibb Institute for Medical Research, New Brunswick, N.J.

1 Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

TABLE I.

Development of Penicillin Resistant Strains of *Staphylococcus aureus in vitro*. In all strains there was visible subculture growth.

Strain	Days exposure to penicillin	Concentration of Penicillin in Florey units per cc									
		5.71	2.85	1.42	.71	.35	.17	.08	.04	.02	.01
Aurice	0	0	0	0	0	0	0	0	0	+	+
							0	0	+	+	+
	24	0	0	0	0	+	+	+	+	+	+
			0	0	0	+	+	+			
	38	0	0	+	+	+	+	+	+	+	+
		0	0	+	+	+	+				
	54	0	+	+	+	+	+	+	+	+	+
			+	+	+	+					
Ram	0	0	0	0	0	0	0	0	0	+	+
							0	0	0	+	
	30	0	0	0	0	0	+	+	+	+	+
					0	+	+	+	+		
	41	0	0	0	+	+	+	+	+	+	+
		0	0	0	0	+	+	+			
	49	0	0	+	+	+	+	+	+	+	+
		0	0	+	+	+	+	+			

TABLE II.

Development of Resistant Strains of *Staphylococcus aureus* During Penicillin Therapy. In all strains there was visible subculture growth.

Case	Diagnosis	No. days treated	Total Penicillin administered (Florey units)	Concentration of Penicillin in Florey units per cc									
				5.71	2.85	1.42	.71	.35	.17	.08	.04	.02	.01
J.B.	Chronic osteomyelitis	0	0	0	0	0	0	0	0	0	+	+	+
									0	0	+		
		18	540,000	0	0	0	+	+	+	+	+	+	+
				0	+	+	+	+	+				
J.R.	Empyema	4	50,000	0	0	0	0	+	+	+	+	+	+
					0	+	+	+	+				
H.H.	,,	0	0	0	0	0	0	0	0	0	0	+	+
									0	0	0	+	
		62	111,000	0	+	+	+	+	+	+	+	+	+
				0	+	+	+						
A.P	Axillary abscess	0	0	0	0	0	0	0	0	0	0	0	+
									0	0	0	0	+
		2	10,000	0	0	0	0	+	+	+	+	+	+
						0	0	0	+	+	+	+	+

to penicillin. Strain Ram showed a similar degree of increased resistance. It is of some interest that a strain of hemolytic streptococcus exposed to penicillin for a period of 45 days did not become "penicillin-fast."

Of importance are the observations made on the strains of staphylococci isolated from 14 patients during the course of penicillin therapy.‡ In only 4 subjects was a change in the susceptibility of the organism demonstrated. The results of these observations are recorded in Table II.

J.B., a 47-year-old male with chronic osteomyelitis of the left femur was treated by intravenous injections of penicillin. Cultures of the draining sinus were taken daily, and after 18 days of therapy the resistance of the staphylococcus to the action of penicillin had increased 64-fold. In this subject the local lesion was not sterilized by penicillin therapy.

Patient J.R., a male aged 16 years, de-

‡ Details of the results obtained with penicillin therapy will be reported at a later date.

veloped empyema following a left lower lobectomy. In this subject the strain of *Staphylococcus aureus* isolated prior to treatment was not tested; however, the organism isolated 4 days after the institution of local penicillin therapy was found to be markedly resistant. A concentration of 2.85 Florey units per cc was necessary to sterilize a culture of this strain.

H.H., a male aged 14 years, received 18 injections of penicillin in 62 days into a chronic empyema cavity. The original strain of *Staphylococcus aureus* was killed in broth cultures by a concentration of 0.04 Florey unit per cc, whereas the organism isolated on the 62nd day required 5.71 Florey units to effect killing. It was possible to keep the empyema cavity sterile by frequent injections of penicillin.

In one subject (A.F.) with a deep axillary abscess the local injection of penicillin into the infected area did not result in sterilization. In this instance the strain of staphylococcus increased its resistance to penicillin about 16-fold in 2 days.

Discussion. Previous reports have shown that the *Staphylococcus aureus* may be rendered resistant to the action of the sulfonamide drugs[2] and to tyrothricin[3] by growing the organism in increasing concentrations of the two substances. There is also evidence that this organism may acquire resistance in patients with staphylococcal infections receiving either sulfonamide or tyrothricin therapy.[3,4] It has been suggested that the adaptation of the infecting bacteria to the antibacterial agent may explain certain therapeutic failures.[3,4]

In this study 29 strains of *Staphylococcus aureus* were found to vary only slightly in their susceptibility to the action of penicillin. Twenty-five of the 29 strains were killed in broth cultures by a concentration of 0.04 to 0.08 Florey unit per cc. This suggests that the variation in susceptibility of different strains of *Staphylococcus aureus* does not play an important role in the therapeutic effectiveness of penicillin therapy in man.

Florey *et al.*[5] were able to produce penicillin-"fast" strains of *Staphylococcus aureus* by cultivation in broth in the presence of increasing concentrations of penicillin. A 30-fold resistance was produced after 9 weeks' exposure to penicillin. These *in vitro* observations were confirmed by the present studies. Two strains were rendered resistant 64-fold in a period of 54 and 49 days. It is of interest that the adaptation of *Staphylococcus aureus* to the antibacterial effect of penicillin takes place only after a prolonged exposure whereas sulfonamide resistance is produced much more readily.[2]

The nature of the acquired resistance of *Staphylococcus aureus* is not known. Florey showed that there was a reduction in the growth-velocity of the resistant strains.[5] No penicillin-destroying enzyme was demonstrated in the resistant strains.[5] Gramicidin-resistant and non-resistant staphylococcic strains were found to be equally susceptible to the action of penicillin.[6] Powell and Jamieson[7] observed that sulfonamide-fast pneumococci were susceptible to the antibacterial effects of penicillin.

In the present studies 4 relatively resistant strains of *Staphylococcus aureus* were isolated from patients during the course of penicillin therapy. In 3 subjects the organism isolated before the institution of therapy was compared with a later strain and found to be much less resistant. This shows, therefore, that in certain subjects penicillin therapy may result in the development of resistant strains. In one subject (J.B.) such resistance was believed to be a factor in the poor results obtained.

Conclusions. Strains of *Staphylococcus aureus* vary only slightly in their susceptibility to the antibacterial action of penicillin. By growing the organism in increasing concentrations of penicillin over a long period it was

2 Strauss, E., Dingle, J. H., and Finland, M., *J. Immunol.*, 1941, **42**, 331.

3 Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 346.

4 Vivino, J. J., and Spink, W. W., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 336.

5 Florey, H. W., Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., and Jennings, M. A., *Lancet*, 1941, **2**, 177.

6 Rammelkamp, C. H., unpublished observations.

7 Powell, H. M., and Jamieson, W. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 387.

possible to render the organism resistant to penicillin.

Similar degrees of increased resistance were found in 4 strains of staphylococci isolated during the course of penicillin therapy for localized infections.

13987

Prolonged Adrenalin Hypertension and Subsequent Circulatory Failure.*

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Previous reports from this laboratory[1] supported the view that a state of shock which cannot be reversed by substantial infusions of the animal's own blood and which is characterized by pathological changes in the mucosa of the upper intestine can follow hemorrhage; but this only occurs when arterial pressures have been reduced severely for significant periods of time. Diminished blood flow through tissues dominantly due to hypotension seems to be the effective agent. The question remains whether the blood flow through tissues can be throttled to a similar degree of intensive vasoconstriction, while arterial pressures remain essentially normal or even become elevated.

Experimental evidence for this possibility has been sought by two types of investigation, (a) prolonged stimulation of afferent pressor nerves and (b) continued administration of potent vasoconstrictor drugs, notably adrenalin. Results from the use of the former have been consistently negative; those realized by the latter procedure remain contradictory.[2] This is due in part to the fact that prolonged administration of adrenalin causes diverse direct and secondary effects on the heart, circulation and respiration, from which an animal may expire during or following its administration. Among these accidents are pulmonary edema, myocardial insufficiency, ventricular fibrillation and respiratory failure. The possibility also exists that some investigators used doses which were too large and others, doses which were too small.

Methods. In order to compare results with those of hemorrhagic shock, dogs were anesthetized with morphine followed by "just adequate" doses of sodium barbital. Respiration, mean femoral, right atrial and intrathoracic pressures were recorded. Adrenalin chloride (Parke, Davis and Co.) was slowly and continuously infused as a 1:5000 solution into a cannulated femoral vein by means of a Mariotte burette. Table I gives the data from separate experiments. The rate of administration ranged from .0069 to .0398 mg per kilo per minute. The total dose depended partly on the weight of the animal. The duration of the infusions varied from 51 to 120 min.

After cessation of the infusion the animals were observed for 3-5 hr. As a rule, mean arterial pressure declined to, or somewhat below, control levels within 5 to 15 min. Thereafter, three types of sequence were observed: (1) Mean pressure remained above 80 min for 4 to 5 hr. (2) While mean pressure remained reasonably normal, the animal expired suddenly, due to respiratory failure. (3) Mean arterial pressure fell progressively to 40 or 20 mm and the animal expired. Only the latter sequence was regarded as a reasonable criterion of progressive circulatory failure, which was probably irreversible. These are designated by the term, "shock," in Table I. A survey of 10 experiments shows that 2 animals (not included in Table I) died during administration of adrenalin due to causes which had no relation to shock. Two

* This investigation was supported by a grant from the Commonwealth Fund.

1 Werle, J. M., Cosby, R. S., and Wiggers, C. J., *Am. J. Physiol.*, 1942, **136**, 401.

2 Wiggers, C. J., *Physiol. Rev.*, 1942, **22**, 74.

TABLE I.

Exp.	Wt, kilos	Dose, mg/K/min	Total dose, mg	Duration of inj. (min)	Mean pressure during inj. (approx.) mm Hg	Dynamic outcome	Autopsy findings
KA 76	12	.0215 and .0398	13.2 40	51 84	186-200 156	No shock 3 hr No shock 7 hr	Intestinal mucosa normal. Subendocardial hemorrhagic areas. Lower lung lobes—few hemorrhages. Spleen small.
78	11	.0198	19.5	92	170-200	No shock 5 hrs	Upper gut mucosa moderately congested. Pleural and sub-endocardial hemorrhagic spots. Some brownish pericardial fluid. Spleen contracted.
79	11.5	.0218	17.4	70	240-210	No shock 4 hr	Upper intestinal mucosa negative. Ileum slightly congested. Stomach mucosa hyperemic. Profuse pericardial effusion. Subepi- and endocardial hemorrhages.
80	16.5	.0239	30	83	240-200	Fall in B.P. to 40 mm in 72 min. Lived 5⅔ hr. Shock	Intense duodenal congestion and swelling—bloody fluid in lumen. Increased pericardial and pleural fluid. Intense subendocardial hemorrhages. Pulmonary congestion—no edema.
81	9	.0228	24.6	120	260	Died failure of resp. 65 min. Shock (?)	Marked engorgement and edema of duodenal mucosa. Small amount clear pericardial fluid. Profuse subendocardial hemorrhage and apical epicardial hemorrhages.
82	9	.0181	20	120	140-180	Died shock 2 hr	Few petechial hemorrhages in duodenum and slight congestion. Large amount of brownish pericardial fluid. Diffuse epi- and pericardial hemorrhages.
84	7	.00694	3.43	70	180+	No shock 3 hr	Duodenal mucosa moderately congested. Small amount pericardial and pleural fluid. Few small subendocardial hemorrhagic spots.
85	6	.018	13	96	220-80	Slight hypotension 70 mm in 2 hr. No shock	Very slight or questionable duodenal congestion. Some pericardial effusion. Few hemorrhagic spots on epi- and endocardium.

animals (80, 82), and questionably a third (81) showed definite circulatory signs of shock. In 5 experiments (76, 78, 79, 84, 85), mean arterial pressure never fell below 80 mm for 3 to 7 hr after discontinuance of the adrenalin infusion.

Necropsies were made on all animals and the most conspicuous changes are recorded in Table I. This serves to indicate that extensive hemorrhagic lesions occurred fairly generally in all animals regardless of the course. Their distribution was far more general than has ever been observed in hemorrhagic shock. Involvement of the endocardium, epicardium, and sometimes the liver was far more common. Small pericardial and pleural effusions were frequently present. However, the duodenal mucosa which showed such characteristic changes in hemorrhagic shock was negative in 3 dogs (76, 79, 85), moderately congested in 3 animals (78, 82, 84) and intensely congested or hemorrhagic in only 2 dogs (80, 81). These observations, correlated with the dynamic course, indicate that prolonged vasoconstriction by adrenalin *can cause* damage to smaller vessels, but its action is by no means as selective as appears to be the case in hemorrhagic shock. Even if we add together the animals which showed *either* a progressive downward trend of blood pressure *and/or* significant intestinal changes (78, 80, 81, 82, 84), prolonged administration of adrenalin gave dynamic or pathological evidence of an impending irreversible state in only half of our animals (total 10 dogs).

Conclusion. In about 50% of our experiments prolonged administration of adrenalin (.00694 to .0398 mg kilo/min.) caused 2-7 hr after cessation of infusion either dynamic evidence of irreversibility or postmortem changes in the upper intestine which resembled those of hemorrhagic hypotension. Adrenalin constriction can lead to shock, but does not unfailingly do so. Our experiments have no bearing on the problems, whether equally intensive generalized constriction occurs in other types of clinical or experimental shock or whether lesser degrees of vasoconstriction can cause similar consequences.

13988 P

Reactions of Monkeys to Experimental Respiratory Infections. VI. Spontaneous and Experimental Infections in Nutritional Deficiency States.*

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Clinical observations have indicated that patients affected with certain deficiency diseases are prone to develop infections. Experimental data in support of the apparent relationship of B-avitaminosis to susceptibility to infection in monkeys have been accumulating in recent years.[1-6]

With increased knowledge of the components of the vitamin B complex, experimental diets more accurately known and controlled with respect to vitamin fractions than those previously used, are now available. Our interest has been not only to study spon-

* This work has been aided by a grant from the International Health Division of the Rockefeller Foundation. Constituents of the special diets were generously furnished by the S.M.A. Corporation.

1 Langston, W. C., Darby, W. J., Shukers, C. F., and Day, P. L., *J. Exp. Med.*, 1938, **68**, 923.

2 Janota, M., and Dack, G. M., *J. Infect. Dis.*, 1939, **65**, 217.

3 Topping, N. H., and Fraser, H. F., *U. S. Public Health Rep.*, 1939, **54**, 416.

4 Tomlinson, T. H., *U. S. Public Health Rep.*, 1939, **54**, 431.

5 Day, P. L., Langston, W. C., Darby, W. J., Wohlin, J. C., and Nims, V., *J. Exp. Med.*, 1940, **72**, 463.

6 Chapman, O. D., and Harris, A. E., *J. Inf. Dis.*, 1941, **69**, 7.

taneous infections as they developed under these conditions, but also to follow the measurable resistance to selected infectious agents when experimentally introduced.

The experiments previously reported[7] from these laboratories were conducted simultaneously with monkeys from the same stock but maintained on a complete diet, and furnish a basis for comparison.

Methods. The diets used and the hematologic changes noted have been described in the preceding paper.[8] The monkeys (*Macaca mulatta*) were isolated in individual cages for 2 to 3 weeks preceding the experiments and throughout the course of the studies. Daily clinical observations and periodic stool cultures were made. Serologic and bacteriologic examinations were conducted when indicated.

Results. Eleven monkeys developed spontaneous infections while in a poor nutritional state and died between the 23rd and 180th diet day. Monkey No. 119, receiving the basic diet with only ascorbic acid as supplement, died in 23 days with dysentery. Monkeys No. 4 and 11 on diet 1 and monkeys No. 33, 42, 66, 136 and 146 on diet 2 succumbed with dysentery on the 107th, 85th, 122nd, 120th, 180th, 154th and 95th days respectively. *Shigella dysenteriae* (Flexner) was isolated from the stools during life from monkeys No. 33, 66, 136 and 146 and was cultured from the scrapings of the ulcerated mucosa of the colon at necropsy in all cases. No dysentery occurred in any animals on normal diets.

Monkey No. 8 on diet 600 exhibited marked ulcerations of the gingiva and buccal and labial mucosa, as well as anorexia, diarrhea and general malaise. During the last few hours of life, the animal's condition changed precipitously from a state of fair activity in the morning to one of complete prostration in the late afternoon. Postmortem findings included the recovery of hemolytic *Staphylococcus aureus* from the spleen, kidney, liver, blood and peritoneal fluid. The primary focus of infection was probably the markedly ulcerated upper lip which yielded *Staphylococcus aureus* on previous culture. All monkeys on the 600 diet developed lesions of the oral mucous membranes which often became infected with streptococci, staphylococci or the flora of Vincent's angina. In monkeys on normal diets, no such lesions appeared spontaneously, and when accidental trauma of gingival or buccal mucosa occurred, no secondary infections were observed.

Monkey No. 131 after 31 days on diet 1 succumbed to pneumonia due to *Klebsiella pneumoniae.* This organism was found occasionally in throat cultures of our stock monkeys. Monkey 41, kept on diet 2 for 124 days, died with an intestinal infestation with *Trichomonas intestinalis* and pneumonia due to *Streptococcus hemolyticus,* Group C. In addition, it exhibited facial edema.

Of 6 monkeys in a deficient state and inoculated intranasally, under light ether anesthesia, with *Streptococcus hemolyticus,* Group C, 5 (No. 23 on diet 1, Nos. 51, 54, and 55 on diet 2, and No. 14 on diet 600), developed specific infections ending fatally 7 to 13 days after inoculation. Monkey No. 23 (Fig. 1) was inoculated on the 154th diet day at the time of a nutritionally induced leukopenia (W.B.C. 3000, neutrophils 800). A typical but abortive leukocytic response to infection (W.B.C. 10,000, neutrophils 8800) was promptly followed by exhaustion of marrow reserves (terminal W.B.C. 1600, neutrophils 880). (Contrast with Fig. 1, Ref. 7, p. 562). Of the 5 monkeys dying of streptococcus septicemia, 3 had facial erysipelas that yielded the same organism. Precipitin, antistreptolysin and complement titers, as well as opsonocytophagic activity, were similar to those found in monkeys on adequate diets[7] similarly treated.

Of 7 monkeys inoculated intranasally, under light ether anesthesia, with the PR-8 strain of influenza virus A, 5 (No. 27 on diet 1, Nos. 53, 56, 69 on diet 2, and No. 24 on diet 600) died 2, 8, 11, 7 and 3 days, respectively, after inoculation. Small peribronchial areas of consolidation were observed at necropsy, and virus was recovered from fil-

[7] Woolpert, O. C., Schwab, J. L., Saslaw, S., Merino, C., and Doan, C. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 558, 560, 563, 566.

[8] Wilson, H. E., Doan, C. A., Saslaw, S., and Schwab, J. L., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 341.

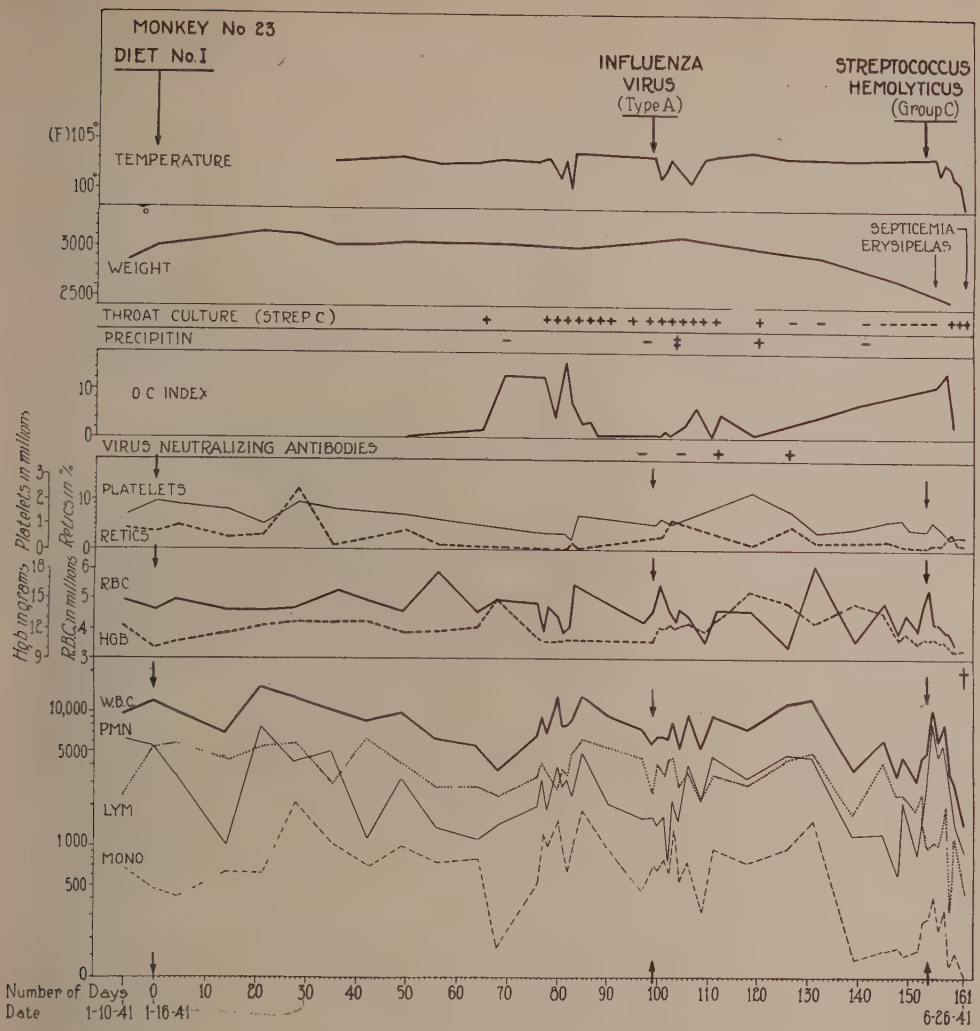

FIG. 1.

trates of the lungs of 3 animals. Neutralizing antibodies were demonstrable in the antemortem serum of M. 56 (8th day) and M. 69 (11th day). In normal monkeys so treated the antibody was detected at about the same interval.

To date 2 monkeys of 32 maintained on an adequate diet and inoculated intranasally with hemolytic streptococci and influenza virus or with influenza virus alone have died from the experimentally induced infection. By contrast, 10 of 13 monkeys on the dietary regimes indicated have died following inoculation with the same agents.

None of the above spontaneous infections was observed over a period of 3 years in an average population of approximately 25 monkeys (total 100) maintained on a stock diet.

Summary. Monkeys on a vitamin B-free basic diet, supplemented by riboflavin, thiamine chloride, nicotinic acid amide, calcium pantothenate, pyridoxin hydrochloride, sodium paraminobenzoate, choline chloride, pimelic acid, glutamine and inositol, de-

veloped a striking granulopenic leucopenia, and manifested a markedly lowered clinical resistance to spontaneous infections with high mortality. The susceptibility to experimental infections with *Streptococcus hemolyticus*, Group C, and to influenza virus A, administered intranasally, was likewise increased in contrast with the controls on normal diet.

13989

Nutritive Value of Keratins. I. Powdered Swine Hoofs.*

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Routh and Lewis[1] recently summarized the literature dealing with the indigestibility of keratins in their natural state. In the same paper they reported that wool which had been powdered in a steel ball mill was readily hydrolyzed *in vitro* by pepsin and trypsin. In subsequent studies Routh[2] found that rats were able to use powdered wool as a source of protein for growth when the material was supplemented with tryptophane, methionine, histidine, and lysine.

The results of these investigations on wool suggested the possibility of converting keratin waste such as horns, hoofs, hair, and feathers into animal feed. This report deals with studies on the nutritive value of powdered swine hoofs fed as a source of protein in the diet of rats and chicks.

Mixed hog hoofs collected at a packing house were ground to a fine, gray powder until all the material passed through a 60-mesh screen.‡ The nitrogen content of the material was 14.5% and it was compared on a weight basis with casein and cartilage without regard to differences in protein content.

Groups of male albino rats 21-22 days of age and weighing 40-50 g were fed the experimental diets given in Table I for 4 weeks. Water and feed were supplied *ad libitum* and body weights were recorded weekly. Typical gains obtained on the different diets are also given in Table I.

Powdered hoofs fed at a level of 18-20% in the diet of rats failed to produce rates of gain comparable to those obtained with equal weights of casein. However, when the level was increased to 30%, growth nearly equal to that of rats fed 18% casein was obtained. Some supplemental effect was obtained when the two proteins were fed together and it is of interest to note that better growth resulted when 15% powdered hoofs were fed with 5% casein than when 9% of powdered hoofs were fed with 9% casein.

The composition of the diets used for the chicks is given in Table II. Day-old White Leghorn chicks were fed the diets for a period of 4 weeks. At the end of 4 weeks the chicks were killed and the gizzards examined for possible lesions.

Powdered hoofs fed at the level of 24% in the diet of chicks produced a higher growth rate and a better development of feathers than an equal percentage of casein. Although the growth rate obtained when 18% casein was supplemented with 10% powdered hoofs was less than that obtained on the diet containing a 10% supplement of cartilage, it was appreciably more than the growth rate obtained with 24% casein. Examination of the gizzards revealed a distinctive condition of the lining in all of the groups fed powdered hoofs. The surface of the linings showed a widespread roughening and cracking. The majority of the fissures were parallel with the normal folds

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

† Wilson and Company Fellow.

1 Routh, J. I., and Lewis, H. B., *J. Biol. Chem.*, 1938, **124**, 725.

2 Routh, J. I., *J. Nutrition*, 1942, **23**, 125.

‡ Prepared by Wilson and Company, Inc.

TABLE I.
Diets and Growth Results in Rat Experiments.

Group	I	II	III	IV	V	VI	VII
	%	%	%	%	%	%	%
Purified casein	18	9	0	20	5	0	0
Powdered hoofs	0	9	18	0	15	20	30
Sucrose	69	69	69	67	67	67	57
Liver powder Fraction B	0	0	0	2	2	2	2
Liver Powder 1-20	2	2	2	0	0	0	0
Avg daily gain in body wt, g	3.6 (3.2-4.2)	2.5 (2.2-3.2)	1.3 (1.0-1.5)	3.6 (3.5-4.0)	3.5 (3.0-3.9)	1.1 (0.4-1.4)	3.2 (3.0-3.5)

All diets contained in addition to the above listed constituents, Salt IV, 4%; corn oil, 5%, cod liver oil, 2%, and vitamin supplement as follows: 2 mg riboflavin, 2 mg thiamine chloride, 2 mg pyridoxine hydrochloride, 1 g choline hydrochloride, and 10 mg calcium pantothenate per kg. Six rats were used in each group.

Liver preparations supplied through courtesy of Wilson Laboratories.

Synthetic vitamins supplied by Merck and Company.

TABLE II.
Diets and Growth Results on Chick Experiments.

Group	I	II	III	IV
	%	%	%	%
Purified casein	24	18	18	0
Powdered hoofs	0	0	10	24
Cartilage	0	10	0	0
Dextrin	58	54	54	58
Avg body wt at 4 wks	94 (50-150)	233 (173-263)	181 (145-225)	121 (93-152)

All the diets contained in addition to above listed ingredients, brewer's yeast, 5%; soy bean oil, 5%; salt IV, 4%; cod liver oil, 2%, and vitamin supplements as follows: 15 mg calcium pantothenate, 3 mg thiamine chloride, 1.5 g choline hydrochloride, 3 mg pyridoxine hydrochloride, 3 mg riboflavin. Twelve chicks were used per group.

Liver preparations supplied through courtesy of Wilson Laboratories.

Synthetic vitamins supplied by Merck and Company.

Yeast supplied by Pabst Brewing Company.

of the linings and did not have the crater appearance of lesions reported by other workers.[3-8] They appear to be similar to the gizzard lesions described by Jungherr.[9]

Discussion: The data on rats and chicks indicate that these animals are able to digest and utilize the protein of powdered hoofs. When 18% and 20% of the diets were supplied by powdered hoofs the protein supply was inadequate for the growth of rats. By increasing the intake of powdered hoofs to 30% of the diet or combining the powdered hoofs with casein an adequate protein source was obtained.

From these facts it appears that one or more amino acids are not available to growing rats in adequate amounts in powdered hoofs. This lack could be due to incomplete digestion of the material or an imbalance of amino acids. With regard to this point it was noted that the group of rats receiving 30% powdered hoofs as the dietary source of protein produced an average daily gain of 3.17 g which greatly exceeds the maximum growth rates reported for powdered wool supplemented with amino acids.[2] From the data of the chick experiments it appears that powdered hoofs alone

3 Holst, W. F., and Halbrook, E. R., *Science*, 1933, **77**, 354.

4 Dam, H., *Nature*, 1934, **133**, 909.

5 Dam, H., and Schönheyder, F., *Biochem. J.*, 1934, **28**, 1355.

6 Almquist, H. J., and Stokstad, E. L. R., *Nature*, 1935, **136**, 31.

7 Bird, H. R., Kline, O. L., Elvehjem, C. A., Hart, E. B., and Halpin, J. G., *J. Nutrition*, 1936, **12**, 571.

8 Almquist, H. J., and Stokstad, E. L. R., *Nature*, 1936, **137**, 581.

9 Jungherr, E., *Conn. Agr. Exp. Sta. Bul.*, 1935, **202**, 52.

are more adequate for the growth of chicks than purified casein alone. Other workers have shown that the chick has a greater requirement for arginine,[10] glycine,[11] and cystine[12] than the rat. Apparently powdered hoofs supply these amino acids in greater amounts than casein. Therefore, the deficiencies of powdered hoofs in the nutrition of the rat may be due to amino acid imbalance rather than indigestibility. However, the latter possibility cannot as yet be eliminated entirely since the digestive tract of the chick may be more capable of digesting material of this nature than the tract of the rat.

Although gizzard lesions were only observed in those chicks receiving powdered hoofs, it appears from other work performed with practical poultry diets that the lesions are not specific for powdered hoofs.

Conclusions: Rats and chicks are able to utilize powdered pig and hog hoofs as a source of protein for growth. The available protein of powdered hoofs is more adequate for the growing chick than for the growing rat.

[10] Arnold, A., Kline, O. L., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1936, **116**, 699.

[11] Almquist, H. J., Stokstad, E. L. R., Mecchi, E., and Manning, P. D. V., *J. Biol. Chem.*, 1940, **134**, 213.

[12] Briggs, G. M., Jr., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1942, **144**, 47.

13990

Titration and Neutralization of the Western Strain of Equine Encephalomyelitis Virus in Tissue Culture.*

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Titration of virus potency and demonstration of viral neutralizing antibodies are procedures not only involving large numbers of animals but also presenting disadvantages which make quantitative interpretation difficult. In an effort to evolve a simplified approach and particularly to remove the variable of individual animal reactivity, tissue culture methods were utilized for titration and neutralization of the Western strain of equine encephalomyelitis virus (W.E.E.). The method is presented here. The principle on which the study was made possible is based on the finding that tissue failed to grow when it was heavily infected with the virus.

In Vitro Titration. Ten-fold dilutions of W.E.E. virus were made in a buffered salt solution. Instead of injecting the virus into animals, one drop of the material from each dilution was inoculated into tissue cultures consisting of a series of tubes each containing 10 pieces of minced skeletal muscles from a 9-day developing chick embryo and 1 cc of serum ultrafiltrate diluted in buffered salt solution.[1] The different mixtures were incubated at 37.5°C. At the end of 48 hours of incubation, pieces of tissue from each tube were transferred and patched with plasma in micro-culture slides. They were then kept at 37.5°C and the readings were made under the low power microscope 48 hours later. It was found that cells which were not infected (or had overcome the infection) grow out in the plasma patch. Growth was abundant and sheets of fibroblasts were clearly visible. Contrariwise, when virus was present, no growth from the explant was observed.

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† The author wishes to express his appreciation to Dr. M. Sanders, Dr. A. R. Dochez and Dr. W. W. Palmer for their constant encouragement and interest.

[1] Simms, H. S., and Sanders, M., *Arch. Path.*, 1942, **33**, 619.

TABLE I.
Comparison of *in vitro* and *in vivo* Titration of W.E.E. Virus.

Titrated in	Virus dilution tested							
	No virus	10-2	10-3	10-4	10-5	10-6	10-7	10-8
Mice	A A A	2 2 2	2 2 2	2 2 2	2 3 3	A A A	A A A	A A A
Tissue culture	G G	0 0	0 0	0 0	0 0	0 0	G G	G G
	G G	0 0	0 0	0 0	0 0	0 0	G G	G G

A = Mouse remained alive 5 days.
Each number indicates day of death of the animal after the infection and presumably represents a virus death.
G = Growth of fibroblasts from explants noted.
0 = No growth from the explants.
Each "G" or "O" represents growth activity of a single explant. Four explants were used for each plasma preparation.

To prove that the tissue was really infected after this period of incubation, the supernatant fluid of the cultures inoculated with different dilutions of the virus was injected intracerebrally into mice soon after the pieces of tissue had been removed from the tubes to the plasma preparations. As was to be expected whenever the tissue was infected, the virus was detected in the supernatant fluid by mouse test.

The sensitivity of the *in vitro* titration method presented here was compared with intracerebral mouse inoculation and the results shown in Table I indicate that the titre is higher in the *in vitro* test than in the *in vivo* experiment.

The test was repeated 3 times with similar results.

In Vitro Neutralization. A volume of 10-fold dilutions of the virus was mixed with an equal amount of hyperimmune anti-W.E.E. horse serum prepared by Lederle Laboratories, Inc., New York, N.Y. For control, anti-meningococcus horse serum was used. The serum-virus mixtures were incubated in the water bath at 37.5°C for 30 minutes. Two drops from each mixture were then introduced into separate tubes each containing about 15 pieces of tissue. The serum-virus mixtures and tissue were incubated at 37.5°C for 15 minutes in order to allow the virus to get into the cells. The tissue was then washed with buffered salt solution for 3 times, each time with 5 cc of the solution. The washed tissue from each tube was then transferred into separate tubes each containing 1 cc of dilute serum ultrafiltrate. The entire preparation was incubated at 37.5°C. At the end of 48 hours of incubation the tissue was patched with plasma and returned to the incubator. Readings were made 48 hours later under the low power microscope.

Again the activity of the virus in tissue culture was at all times compared and confirmed by mouse inoculation. The results of the comparison of the *in vitro* and the *in vivo* neutralization experiments are shown in Table II. It can be seen that the amount of neu-

TABLE II.
Comparison of the *in vitro* and *in vivo* Neutralization Tests with W.E.E. Virus.

Serum used	Titrated in	Dilution of the virus tested							
		10-0	10-1	10-2	10-3	10-4	10-5	10-6	10-7
Immune	Mice	2 2	A A	A A	A A	A A	A A	A A	
		2 2	A A	A A	A A	A A	A A	A A	
	Tissue	00000	00000	GGGGG	GGGGG	GGGGG	GGGGG	GGGGG	
	culture	00000	00000	GGGGG	GGGGG	GGGGG	GGGGG	GGGGG	
Control	Mice			2 2	2 2	2 2	A A	A A	A A
				2 2	2 2	3 3	A A	A A	A A
	Tissue			00000	00000	00000	00000	GGGGG	GGGGG
	culture			00000	00000	00000	00000	GGGGG	GGGGG

Legends as in Table I except that 10 explants were used for each plasma preparation.

tralization (10,000 neutralizing doses) in the *in vitro* experiment is the same as that observed by animal inoculation. Apparently the scale of sensitivity for the detection of virus in both the titration and neutralization experiments has shifted one dilution higher than the *in vivo* tests. The possibility must also be considered that the shift in neutralization activity measured by the *in vitro* method may to some extent be a manifestation of reactivation of neutral mixtures by dilution.[2] Further study of *in vitro* neutralization technic should clarify this point.

Improvement of technical details is being attempted and will be further described.

Conclusions. Titration of potency of W.E.E. virus and quantitative estimation of neutralizing antibodies in hyperimmune horse serum have been demonstrated by use of tissue cultures.

The *in vitro* method of detecting the presence of virus appeared to be more sensitive than intracerebral mouse inoculation.

2 Pierce, M. E., Kempf, J. E., and Soule, M. H., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 604.

13991

Choline Esterase Content of Tissues Without Innervation (the Placenta).

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The probable importance of the choline esterase-acetylcholine system to transmission in the nervous and neuromuscular system suggested that the choline esterase content of the placenta, an organ without nervous tissue (Schmitt[1]) should be investigated. Other tissues without innervation are the blood and the denervated muscle. The choline esterase content of denervated muscles decreases for some weeks after denervation but does not disappear completely as shown in the rat by Martini and Torda[2,3] and Meng[4], in the dog by Martini and Torda[5], in the frog by Feng and Ting[6], in the rabbit by Leibson[7] and in the guinea-pig by Couteaux and Nachmansohn.[8]

The human placenta has a rather high acetylcholine content (Chang and Gaddum,[9] Chang and Wong[10], Chang,[11,12] Reynolds and Foster,[13] Cattaneo[14]). Changes in acetylcholine sensitivity (Euler[15]) and acetylcholine content (Chang,[12,16], Chang, Lee, Meng and Wong[17]) of the placenta during different experimental conditions suggest that this tissue contains choline esterase.

Method. Some minutes after normal delivery the human placenta was perfused through the umbilical vein until all the blood was removed. The choline esterase content of 100 mg finely ground tissue was determined

* Now at Cornell University Medical College, New York.

1 Schmitt, *Z. Biol.*, 1922, **75**, 19.

2 Martini and Torda, *Klin. Wschr.*, 1937, **16**, 824.

3 Martini and Torda, *Klin. Wschr.*, 1938, **17**, 97.

4 Meng, *Chin. J. Physiol.*, 1940, **15**, 143.

5 Martini and Torda, *Boll. Soc. Ital. Biol. Sper.*, 1938, **13**, 1056.

6 Feng and Ting, *Chin. J. Physiol.*, 1938, **13**, 141.

7 Leibson, *Bull. Biol. Med. Exp. U. R. S. S.*, 1939, **7**, 517.

8 Couteaux and Nachmansohn, PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 177.

9 Chang and Gaddum, *J. Physiol.*, 1933, **79**, 255.

10 Chang and Wong, *Chin. J. Physiol.*, 1933, **7**, 151.

11 Chang, PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 665.

12 Chang, *Chin. J. Physiol.*, 1938, **13**, 145.

13 Reynolds and Foster, *Am. J. Physiol.*, 1939, **127**, 343.

14 Cattaneo, *Arch. internat. Physiol.*, 1933, **37**, 58.

15 Euler, *J. Physiol.*, 1938, **93**, 129.

16 Chang, PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1001.

17 Chang, Lee, Meng and Wang, PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 380.

by Glick's method.[18] Four samples were taken from widely separated parts of each placenta. To 100 mg tissue 0.92 cc carbon dioxide-free distilled water and 1.6 cc veronal buffer solution containing 0.5% acetylcholine chloride were added. The mixture was kept at 35°C for 3 hours. Five cc of physostigmine-brom-thymol blue solution were then added and the mixture titrated with N/10 hydrochloric acid. Each mm of hydrochloric acid corresponds to 18 γ acetylcholine decomposed. Acetylcholine solutions without tissue and tissue without acetylcholine were treated in the same way. The difference between the hydrochloric acid used in the control experiments and that used with the samples containing tissue and acetylcholine corresponds to the acetylcholine which was decomposed by the tissue choline esterase. The choline esterase activity of placenta was calculated from the amount of hydrochloric acid used according to the formula given by Glick.[19]

Results. The choline esterase activity of 25 human placentæ was determined. The values are summarized in Table I.

TABLE I.
Choline Esterase Activity of Human Placentæ After Normal Delivery.
(The value is given in 1/10³ γ, per sec. per mg tissue.)

	Choline esterase activity	
No. of exper.	Mean	S.E.*
25 (4 samples each)	6.0	±0.27

*S.E. of mean $= \sqrt{\frac{\Sigma(\Delta)^2}{N(N-1)}}$

Σ = summation.
Δ = deviation of each sample from the mean.
N = 100.

Discussion. The results of this study show that 1 mg of placenta is able to decompose 6/10³ γ acetylcholine at 35°C in 1 sec. This activity is presumably due to the choline esterase fixed in the intracellular spaces because the prolonged perfusion would wash out much of the extracellular choline esterase. The data for the choline esterase content of the cells given above represent minimum values, since no correction has been made for the extracellular fluid and, further, prolonged perfusion produced an edema and therefore an increase of the weight of the tissue. The presence of choline esterase in the placenta is not due to the choline esterase activity of traces of blood left, a possibility that was excluded by examination of the tissue. Moreover, the samples taken from different parts of the placenta had the same choline esterase activity, even though the maternal and fetal blood differ significantly in choline esterase (Ammon and Voss,[20] Navratil,[21], Torda[22]).

A comparison of the choline esterase content of placenta with the data in the literature for other tissues is difficult because the different methods of determination do not give comparable values. An evaluation may be attempted by comparing the choline esterase content of tissues determined with Glick's method and also with other methods. The choline esterase content of the placenta is about one-tenth that of the choline esterase content of the mucosa of pig pylorus (Glick[23]). By comparing the choline esterase content of the pylorus and serum of pig (Glick, Lewin and Antropol[24]) and that of pig and human serum (Stedman and Stedman,[25] Hall and Lucas[26]) it appears that the choline esterase activity of the maternal blood is about twice as great as that of placenta. If this is the case it is reasonable to assume that the choline esterase content of the placenta originated from the blood by an exchange.

The choline esterase may be distributed equally or may be concentrated in the epithelium of the chorionic villous tissue where, according to Wen, Chang and Wong,[27] the acetylcholine is liberated. In the latter case

18 Glick, *J. Gen. Physiol.*, 1938, **21**, 289.
19 Glick, *J. Gen. Physiol.*, 1938, **21**, 439.
20 Ammon and Voss, *Pflüger's Archiv*, 1935, **235**, 393.
21 Navratil, *Geburtshilfe*, 1937, **114**, 146.
22 Torda, *Biochemica e Terapia Sper.*, 1938, **25**, No. 12.
23 Glick, *J. Gen. Physiol.*, 1938, **21**, 297.
24 Glick, Lewin and Antropol, PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 28.
25 Stedman and Stedman, *Biochem. J.*, 1935, **29**, 2107.
26 Hall and Lucas, *J. Pharm. Exp. Therap.*, 1936, **61**, 10.
27 Wen, Chang and Wong, *Chin. J. Physiol.*, 1937, **10**, 559.

the choline esterase concentration of the chorionic villous tissue would be much greater than that of the blood.

The results of this study suggest that the presence of choline esterase is a generalized phenomenon and is not necessarily related to the presence of nerve elements.

Summary. 1. The choline esterase activity of 25 perfused human placentæ was determined by the method of Glick. 2. The choline esterase contained in 1 mg tissue is able to decompose at 35°C in the average $6/10^3$ γ acetylcholine per sec.

13992 P

Mechanism of the Colloidal Gold Reaction of Blood Serum in Liver Disease.

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When a colloidal gold suspension is added to certain dilutions of blood serum from patients with liver disease flocculation of the colloidal gold occurs in one or more of the first serial dilutions.[1,2] The colloidal gold reaction of blood serum in liver disease has proven a sensitive method of detecting early liver involvement according to Loew and Noth,[3] Mateer *et al.*,[4] and Sweet, Gray and Allen.[5]

Flocculation of the colloidal gold by serum from patients with liver disease does not depend primarily upon a quantitative increase in globulin or upon an inverted albumin-globulin ratio.[1,2] It was suggested by the author[1] that the mechanism of this reaction in liver disease might depend upon a qualitative variation within the serum globulins rather than upon a quantitative change in the total serum globulin.

Electrophoretic studies of the serum proteins in all types of liver disease, both acute and chronic, revealed that the most characteristic and consistent alteration of the serum proteins was an increase in the gamma globulin associated with a decrease in serum albumin.[6]

Since globulin promotes colloidal gold flocculation and albumin protects the colloidal suspension from flocculation[7] these studies were undertaken to investigate the effect of the purified protein fractions, prepared by Dr. Edwin J. Cohn,[8] upon the serum colloidal gold reaction.

Method. Increasing amounts of electrophoretically pure human gamma globulin, albumin, and a purified fraction containing alpha and beta globulins were added to normal blood serum. The serum was then diluted 1:350 with 0.9% sodium chloride and the blood serum colloidal gold reaction was performed in the usual manner. Five cc of properly acidified colloidal gold were added to each of 3 tubes containing 1 cc of serum in final dilutions of 1:3500, 1:7000 and 1:14,000. The tubes were allowed to stand at room temperature and were read after 24 hours. Color changes were expressed by the following numbers: 0—red orange, 1—reddish blue, 2—orchid, 3—blue, 4—light blue, and 5—colorless. Complete flocculation in one or more of the 3 tubes designates a positive test. The usual negative reaction is 222 or 332, and the positive test is 532, 553, or 555.

[1] Gray, S. J., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 470.

[2] Gray, S. J., *Arch. Int. Med.*, 1940, **65**, 524.

[3] Loew, E. R., and Noth, P., *Proc. Am. Physiol. Soc.*, 1941, 176.

[4] Mateer, J. G., Baltz, J. I., Marion, D. F., Hollands, R. A., and Yagle, E. M., *Am. J. Digest. Dis. and Nutrition*, 1942, **9**, 13.

[5] Sweet, W. H., Gray, S. J., and Allen, J. G., *J. A. M. A.*, 1941, **117**, 1613.

[6] Gray, S. J., and Barron, E. S. G., *J. Clin. Invest.*, in press.

[7] Reznikoff, P., *J. Lab. and Clin. Med.*, 1922, **8**, 92.

[8] Cohen, E. J., *Chem. Rev.*, 1941, **28**, 395.

Results. The addition of 0.2 g% of electrophoretically pure human gamma globulin to normal blood serum produced a positive colloidal gold test—532—which increased in intensity as the concentration of gamma globulin was increased to 1.0 g%. At this point all 3 tubes show complete flocculation—555.

Similar amounts of the purified fraction containing alpha and beta globulin were added to normal blood serum and produced no effect whatever on the blood serum colloidal gold reaction.

Pure albumin inhibited the serum colloidal gold reaction. The addition of 0.5 g% of albumin to normal blood serum inhibited the serum colloidal gold reaction from 322 to 111. Increasing the concentration of albumin to 1.0 or 2.0 g% produced complete inhibition of the serum colloidal gold reaction—000.

No significant alteration of pH was observed in the blood sera containing the purified protein fractions.

Summary. The addition of pure gamma globulin to normal blood serum produced a positive serum colloidal gold reaction. The purified fraction containing alpha and beta globulins produced no effect. Albumin inhibited the serum colloidal gold reaction. Since the most characteristic and consistent alteration of the serum proteins in liver disease is a decrease in serum albumin and a relatively large increase in gamma globulin it is suggested that the mechanism of the colloidal gold reaction in liver disease depends upon a relative increase in the gamma globulin of the blood.

The author wishes to express his gratitude to Dr. Edwin J. Cohn for supplying the purified protein fractions used in this study, which were prepared under contract with the Office of Scientific Research and Development upon recommendation by the Committee on Medical Research.

13993 P

Studies on the Mechanism of the Spinal Fluid Colloidal Gold Reaction.

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Zsigmondy[1] laid the foundation for the diagnostic use of the colloidal gold reaction by observing that "certain colloids, especially proteins" prevented the precipitation of colloidal gold suspensions by electrolytes. Lange[2] found that proteins within certain dilutions did not prevent but actually caused precipitation. The mechanism of the colloidal gold reaction has been the subject of much investigation. Numerous workers[3,4,5] have concluded that the globulin content of the spinal fluid is the determining factor in the precipitation of colloidal gold and that albumin protects the colloidal suspension from precipitation. Others[6,7] have suggested, moreover, that the individual globulin fractions played an important role in the precipitation of colloidal gold.

It has been impossible, heretofore, to study the effect of the individual globulin fractions on colloidal gold precipitation because these fractions could not be obtained in pure form. Tiselius[8] has shown that the precipitation ranges of the globulin fractions overlap one another grossly and that the euglobulin, pseudoglobulin and albumin fractions prepared by the usual methods of fractional precipitation[9] are mixtures of albumin, alpha,

1 Zsigmondy, R., *Ztschr. f. anal. chem.*, 1901, **40**, 697.

2 Lange, C., *Berl. klin. Wchnschr.*, 1912, **49**, 897.

3 Felton, L. D., *New York State J. Med.*, 1917, **105**, 1170.

4 Weston, P. G., *Am. J. Syph.*, 1919, **3**, 266.

5 Cruickshank, J., *Brit. J. Exp. Path.*, 1920, **1**, 71.

6 Reznikoff, P. J., *Lab. and Clin. Med.*, 1922, **8**, 92.

7 Mellanby, J., and Anwyl-Davies, T., *Brit. J. Exp. Path.*, 1923, **4**, 132.

8 Tiselius, A., *Biochem. J.*, 1937, **31**, 313, 1464.

beta and gamma globulins.

The electrophoretically pure protein fractions prepared by Dr. Edwin J. Cohn[10] presented an opportunity to study the effects upon colloidal gold flocculation of pure human albumin, gamma globulin and the fraction containing alpha and beta globulins.

Method. Increasing concentrations of pure human albumin, gamma globulin and the fraction containing alpha and beta globulins in amounts varying between 0.005 and 1.0 g% were added to normal spinal fluids and to spinal fluids from patients with general paresis. The albumin and the alpha and beta globulins were isoelectric while the gamma globulin was in salt form. Ten experiments were performed with the normal spinal fluids and 5 with the spinal fluids of patients with paresis. The colloidal gold test was performed in the usual manner by the addition of 2.5 cc of colloidal gold solution to 0.5 cc of spinal fluid in serial dilutions of 1:10, 1:20, 1:40 to 1:5120. The tubes were allowed to stand at room temperature and were read after 24 hours. Color changes were expressed by the following numbers: 0—red orange, 1—red blue, 2—orchid, 3—blue, 4—light blue, and 5—colorless.

Results. The addition of pure gamma globulin to normal spinal fluid produced a typical paretic colloidal gold curve—5555432100. Complete flocculation in the first tube was noted when 0.020 g% of gamma globulin was added to normal spinal fluid. The degree of flocculation increased proportionately with the increased concentration of gamma globulin. The addition of 0.20 g% of gamma globulin caused complete flocculation in 7 of the 10 dilutions. (Table I.)

The addition of pure human albumin to the spinal fluid of a patient with general paresis converted the paretic colloidal gold curve to normal. A strongly positive paretic colloidal gold curve was made to appear normal by the addition of 0.8 g% of albumin. The inhibition of colloidal gold flocculation was progressive as the concentration of albumin increased from 0.05 to 0.8 g%.

Summary. The addition of pure gamma globulin to normal spinal fluid produced a typically paretic colloidal gold curve. The degree of flocculation was proportional to the amount of gamma globulin added. Similar concentrations of the fraction containing alpha and beta globulins did not affect the colloidal gold reaction. Albumin inhibited colloidal gold flocculation. The addition of pure human albumin to the spinal fluid of a patient with paresis converted the paretic curve to normal. Since the gamma globulin is increased in the spinal fluid of patients with paresis,[11] the paretic colloidal gold curve probably depends upon the relative increase in gamma globulin.

The author is greatly indebted to Dr. Edwin J. Cohn for supplying the purified protein fractions used in this study, which were prepared under contract with the Office of Scientific Research and Development upon recommendation by the Committee on Medical Research.

9 Howe, P. E., *J. Biol. Chem.*, 1921, **49**, 93.

10 Cohn, E. J., *Chem. Rev.*, 1941, **28**, 395.

11 Kabat, E. A., Moore, D. H., and Landow, H., *J. Clin. Invest.*, 1942, **21**, 571.

TABLE I.

Effect of Purified Protein Fractions on the Colloidal Gold Curve of Normal and Paretic Spinal Fluid.

Amt of protein added (g%)	Normal spinal fluid			Paretic spinal fluid.
	γ globulin	α and β globulins	Albumin	Albumin
0	0000000000	0000000000	0000000000	5553210000
.005	2211000000	0000000000	0000000000	
.01	2221110000	0000000000	0000000000	
.02	5221100000	0000000000	0000000000	
.03	5522110000	0000000000	0000000000	
.04	5553211000	0000000000	0000000000	
.05	5555432100	0000000000	0000000000	5543210000
.10	5555554210	0000000000	0000000000	5321000000
.20	5555555433	0000000000	0000000000	4211000000
.60				1110000000
.80				0000000000

13994 P

Effect of Purified Protein Fractions on Sedimentation Rate of Erythrocytes.

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It has been established by Fahraeus[1] and others[2] that plasma fibrinogen and globulin increase the sedimentation rate of erythrocytes and that albumin retards the rate of sedimentation. Zarday and Farkas[3] modified normal whole blood by adding fibrinogen and globulin and found that the sedimentation rate was increased in proportion to the amount of protein added. The addition of albumin retarded the sedimentation rate. Coburn and Kapp[4] confirmed these results.

Studies of the relationship of the blood proteins to the sedimentation rate have been conducted heretofore with relatively impure protein fractions obtained by fractional precipitation with neutral salts.[5] Tiselius[6] has shown by electrophoretic analysis that these protein fractions are mixtures of albumin and alpha, beta or gamma globulins and observed that quantitative studies with proteins obtained by fractional precipitation were inaccurate. Thus the electrophoretically pure human protein fractions prepared by Dr. Edwin J. Cohn[7] present an opportunity to study the effect of the protein constituents of blood on the sedimentation rate of erythrocytes especially since no investigations have been made of the influence of the various fractions composing the serum globulin on the sedimentation rate of the erythrocytes.

Method. Four purified protein preparations were studied: electrophoretically pure human albumin, gamma globulin and fibrinogen, and a fraction containing alpha and beta globulins. The albumin and alpha and beta globulins were isoelectric, while the gamma globulin and fibrinogen were in the salt form. Increasing amounts of these protein fractions, varying from 0.2 to 2.5% were added to heparinized whole blood, and the sedimentation rates were determined by the Wintrobe[8] method. The pH of the blood samples containing these proteins was unchanged.

Results. Fibrinogen was more effective than any of the protein fractions studied in increasing the sedimentation rate of erythrocytes. The addition of 0.2 g% of fibrinogen to whole blood increased the sedimentation rate from a normal value of 6 to 24 mm in one hour. The sedimentation rate was increased in proportion to the amount of fibrinogen added. The addition of 0.4 g% increased the sedimentation rate to 51 mm and 0.6 g% elevated the sedimentation rate to 54 mm in one hour. Thus the sedimentation rate of erythrocytes may be increased 300 to 800% by the addition of fibrinogen. (Table I.)

The fraction containing the alpha and beta globulins elevated the sedimentation rate less effectively than fibrinogen. The addition of 0.2 g% of this fraction to whole blood increased the sedimentation rate from a normal value of 7 mm to 14 mm in one hour, an increase of 100%. (Table I.) The sedimentation rate was elevated to 18 mm and 25 mm in one hour upon the addition of 0.4 and 0.8 g% respectively of alpha and beta globulins. (Table I.)

Gamma globulin proved least effective in increasing the sedimentation rate. The sedimentation rate in one hour was elevated from a normal value of 5 mm to 8, 11, and 27 mm upon the addition of 0.2 g%, 0.4 g%, and 0.8 g% respectively of pure gamma globulin. The addition of 0.4 g% of gamma globulin to normal blood was required to increase the sedimentation rate 100%. (Table I.)

1 Fahraeus, R., *Acta med. Scandinav.*, 1921, **55**, 1.
2 Kylin, E., *Acta med. Scandinav.*, 1935, **85**, 574.
3 Zarday, I., and Farkas, G., *Z. f. d. ges. exp. Med.*, 1931, **78**, 367.
4 Coburn, A. F., and Kapp, E. M., *J. Clin. Invest.*, 1936, **15**, 715.
5 Howe, P. E., *J. Biol. Chem.*, 1921, **49**, 93, 109.
6 Tiselius, A., *Biochem. J.*, 1937, **31**, 313, 1464.
7 Cohn, E. J., *Chem. Rev.*, 1941, **28**, 395.
8 Wintrobe, M. M., *Internat. Clin.*, 1936, **2**, 34.

TABLE I.
Effect of Pure Protein Fractions on Sedimentation Rate of Blood.

Amt of protein added (g %)	Sedimentation rate (mm in 1 hr)							
	Fibrinogen		α and β Globulins		γ Globulin		Albumin	
0	6	15	7	2	5	10	51	52
.2	24	45	14	3	8	16		
.4	51	50	18	4	11	18	46	
.6	54	56	24	8	17	20	40	50
.8			25	11	27	27	36	
1.0							32	49
2.0							23	40

Albumin inhibited the sedimentation rate of erythrocytes. The addition of 2.0 g% of pure human albumin to normal blood retarded the sedimentation from the original rate of 18 mm to 11 mm in one hour. The inhibition of sedimentation was more pronounced when 2.0 g% of albumin was added to blood with a markedly elevated sedimentation rate; the sedimentation rate was retarded from the original value of 51 mm to 23 mm in one hour.

Summary. Purified fibrinogen increased the erythrocyte sedimentation rate more effectively than the other protein fractions studied; 0.2 g% increased the sedimentation rate 300%. The fraction containing alpha and beta globulins was next most effective; 0.2 g % increased the sedimentation rate 100%. Gamma globulin proved least effective; 0.4 g% was required to increase the sedimentation rate 100%. Pure human albumin inhibited the sedimentation rate of erythrocytes, the inhibition being most pronounced in the presence of a markedly elevated sedimentation rate.

The authors wish to express their gratitudee to Dr. Edwin J. Cohn for supplying the purified protein fractions used in this study, which were prepared under contract with the Office of Scientific Research and Development upon recommendation by the Committee on Medical Research.

13995 P

Milk Influence and Leukemia in Mice.*

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It has been established that an "influence" contained in the breast milk of mice of high mammary cancer strains is important in determining the development of mammary cancer in these stocks.[1,2] This "maternal influence" was originally demonstrated in F_1 hybrid mice representing reciprocal crosses between animals of high and low mammary cancer strains.[3] (F_1 hybrids with mothers of the high mammary cancer strain developed more breast cancer than F_1 hybrids whose mothers were of the low mammary cancer strain.) MacDowell and Richter[4] reported a similar

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1 Bittner, J. J., *Science*, 1936, **84**, 162.

2 Bittner, J. J., *Science*, 1942, **95**, 462.

3 Staff, Jackson Memorial Laboratory, *Science*, 1933, **78**, 465.

4 MacDowell, E. C., and Richter, M. N., *Arch. Path.*, 1935, **20**, 709.

although less marked "maternal influence" with reference to leukemia, the high leukemia C58 strain and the low leukemia StoLi strain being reciprocally crossed in their studies. Foster-nursing experiments failed, however, to demonstrate any "milk-influence." Barnes and Cole[5] observed a decreased incidence of leukemia in mice of a high leukemia stock (Ak) fostered by females of low leukemia stocks (Af and Rf). Fostered animals died relatively early in life, however, from intercurrent disease. Furth, Cole and Boon[6] obtained results similar to those of MacDowell and Richter with respect to the incidence of leukemia in F_1 hybrids, that is, the incidence of leukemia was greater in F_1 hybrids whose mothers were of the high leukemia (Ak) rather than the low leukemia (C3H) stock. The effect was more pronounced in males. It was also found that foster-nursing by low leukemia females significantly lowered the incidence of leukemia in the high leukemia stock. The offspring of the fostered mice, however, developed leukemia to as great a degree as the original leukemic stock. The incidence of mammary cancer, on the other hand, is low in the offspring of fostered mice of a high mammary cancer strain.[7] Mice of resistant strains were rendered susceptible to inoculation of leukemic cells of the Dba strain when foster-nursed by Dba females.[8]

In the experiments being reported here reciprocal crosses to obtain F_1 hybrids were made between strain F mice (high leukemia)[9] and animals of 3 low leukemia strains (CBA, C57 and A).[10] F_1 hybrids were backcrossed to high (strain F) and low (strain A and CBA) leukemia strains in such a manner that strain F influence was supplied entirely by the male or entirely by the female. Observations were also made on F_2 hybrids where the female influence was provided by either high or low leukemia females. In addition, strain F mice were foster-nursed by animals of several low leukemia strains (CBA, I, A, C3H, NH).[10] Transfer of the young to foster mothers was effected within 24 hours after birth. Forty-eight fostered animals have been observed for more than 500 days.

Of the cross F♂ × CBA♀ (or C57♀) 16 out of 33 mice developed leukemia, and in the reciprocal cross, F♀ × CBA♂ (or C57♂) 38 out of 81 developed the disease. Strain CBA breast milk (of the line used in these experiments) carries the milk-influence for mammary cancer. Nine out of 15 female F_1 hybrids (F♂ × CBA♀) with this factor transmitted to them developed mammary cancer, whereas only 4 out of 30 females of the reciprocal cross (F♀ × CBA♂) developed breast cancer.

Out of a group of 12 backcross mice of the genetic constitution F♂ × (F♂ × CBA♀)♀ where there could be no milk influence (if such were present) transmitted from the F strain, a total of 9 mice developed leukemia. This incidence is higher than that of 55% in the pure strain. Of the backcross F♀ × (F♀ × CBA♂)♂ 13 out of 23 mice developed leukemia. Seven out of 19 animals of the backcross CBA♀ × (CBA♀ × F♂)♂ became leukemic. In a more recent study of strain F mice crossed with the A strain, no backcross animals are older than one year of age. Of 41 mice without a possible milk influence for leukemia, F♂ × (F♂ × A♀)♀, 7 have already developed leukemia (the remaining 34 are still alive and vigorous). When F_2 hybrids were obtained by mating F_1 hybrids of the genetic constitution F♀ × CBA♂ (or C57♂) 19 out of 52 animals developed leukemia. When F_1 hybrids of the reciprocal cross were used in breeding for F_2 hybrids 6 out of 11 of the latter became leukemic.

Seventeen of the 48 strain F mice fostered by low leukemia strains have developed leukemia up to the present. Ten are still alive and are more than 500 days of age. Before reaching the age of 400 days 19% of the 48 mice had developed leukemia; 28% of 220 controls developed the disease before 400 days of age. Twenty-eight per cent of fostered mice developed leukemia before the age of 500 days whereas 38% of controls (220 animals) were leukemic before this age. The

[5] Barnes, W. A., and Cole, R. K., *Cancer Research*, 1941, **1**, 99.

[6] Furth, J., Cole, R. K., and Boon, M. C., *Cancer Research*, 1942, **2**, 280.

[7] Bittner, J. J., *Cancer Research*, 1941, **1**, 113.

[8] Law, L. W., *Cancer Research*, 1942, **2**, 108.

[9] Kirschbaum, A., and Strong, L. C., *Am. J. Cancer*, 1939, **37**, 400.

[10] Strong, L. C., *Cancer Research*, 1942, **2**, 531.

total incidence of leukemia in fostered F mice will probably not exceed 40%, whereas the incidence in unfostered strain F mice is 55%.

Summary. Preliminary breeding experiments did not indicate that a specific "milk-influence" (as for mammary cancer) is concerned in the development of leukemia in strain F mice. The incidence of leukemia in a group of 48 strain F mice fostered by low leukemia-strain mice was, however, somewhat lower than in unfostered controls of the same strain.

13996

Pathological Studies of Acute Biotin Deficiency in the Rat.*

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Several groups of workers[1–5] using different types of egg-white rations have reported varying degrees of paralysis in rats. Nielsen and Elvehjem[5] have shown that this paralysis could be prevented and cured by crystalline biotin. The histopathology of the acute form of this deficiency has been studied and is herewith reported.

Methods. The observations which are described at this time were made on tissues from rats in which an acute biotin deficiency had been produced by Nielsen and Elvehjem[5] on their basal egg-white ration. Control rats were maintained on the basal ration supplemented with 1 μg of biotin per day. At the time when the paralysis in the biotin-deficient rats had become extremely severe, the rats were killed and autopsied. Comparable controls were sacrificed at the same time. The thymus, thyroid, liver, adrenal, kidney, testis, epididymis, regions of the denuded skin and leg muscles were fixed in Bouin's fluid and then stained in hematoxylin and eosin. A portion of the spinal cord and 1 sciatic nerve were fixed in 10% formalin buffered at pH 7.0. This portion of the spinal cord and one half of the sciatic nerve were stained by a modified Marchi osmic acid technic.[6] The other half of the sciatic nerve was used for the preparation of frozen sections for observation in the polarizing microscope. Another portion of the spinal cord and the other sciatic were fixed in a fluid consisting of 95% ethanol 100 parts, glacial acetic acid 5 parts, and paraldehyde 2 parts. These tissues were stained by Bodian's silver technic.[7]

Results. The distribution of lesions in the tissues studied is shown in Table I. The thymus grossly appeared much smaller in the biotin deficient animals. Histologically it showed signs of early involution and connective and adipose tissue infiltration.

The testes and epididymi of the deficient animals were very small in proportion to the size of the animal. The tubules of the testis were very small and many signs of a degenerative atrophy were present. There were many atypical spermatids and spermatogonial cells which were being sloughed into the lumina of the tubules. Frequently very large multinuclear cells were seen among the desquamated cells in the lumina. The epithelial cells of the tubules of the epididymis were usually rather high and the lumina were filled with the cells sloughed off in the tubules of the testis.

The skin of the biotin-deficient rats ap-

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

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2 Findlay, G. M., and Stern, R. O., *Arch. Dis. Child.*, 1929, **4**, 1.

3 Parsons, H. T., *J. Biol. Chem.*, 1931, **90**, 357.

4 Salmon, W. D., and Goodman, J. G., *J. Nutrition*, 1394, **8**, 1.

5 Nielsen, E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **144**, 405.

6 Swank, R. L., and Davenport, H. A., *Stain. Tech.*, 1934, **9**, 11.

7 Bodian, D., *Anat. Rec.*, 1936, **65**, 89.

TABLE I.
Number of Rats Which Developed Histopathologic Changes in the Tissues Studied.

Ration	No. of cases	Spinal cord	Sciatic nerve	Thymus	Testis	Epididymis	Skin	Muscle
Basal	20	0	0	12	20	20	20	20
Basal + 1 μg biotin/day	6	0	0	0	0	0	0	0

peared very dry, in some of the cases rather scaly and in all cases there was a very great loss of hair. There appeared to have been an almost complete sloughing of the stratum corneum so that the cornified layer of the epidermis was present in only small areas of the skin. In all cases the stratum Malphigi was rarely more than two cells thick. The hair follicles were filled with cornified debris.

The muscles of the biotin-deficient rats grossly appeared very small and pale. Histologically it was seen that the fibers were atrophied and only faintly striated. There did not seem to be any complete destruction of the fibers or any connective tissue replacement.

In these rats with severely spastic gait, no myelin degeneration was found in either the spinal cord or the sciatic nerves. The axon cylinders and nerve cells also appeared normal.

The thyroid, liver, adrenal and kidney were all normal.

Discussion. The involution of the thymus would appear to be an early manifestation of the ageing process. The degenerative atrophy of the testis and epididymis do not seem to be specifically characteristic of this deficiency since very similar conditions have also been seen in riboflavin[8] and pantothenic acid[9] deficiency.

The normal condition of the spinal cord and peripheral nerves observed in these rats does not agree with the observations of Findlay and Stern[2] who reported a diffuse infiltration of small round cells into the gray matter of the spinal cord and slight but definite myelin changes in the peripheral nerves of rats maintained on an egg-white ration. However these investigators used a ration which was quite low in the vitamin B complex and the lesions observed may have been due to the deficiency of one or more of those factors. It may be, however, that in a more chronic biotin deficiency than that studied by us, some pathology of the nervous system would become evident.

Although the spastic paralysis observed in an acute biotin deficiency is grossly similar to that obtained in riboflavin deficiency,[8] the pathology seems to be different. At present it appears that the paralysis observed in these acutely biotin-deficient rats is not due to a neural lesion.

Summary. The pathology occurring in the thymus, testis, epididymis, skin and muscles of the acutely biotin-deficient rat is reported. No degeneration of the spinal cord or sciatic nerves was observed despite the very extreme spasticity which occurred in many of these acutely deficient animals.

[8] Shaw, J. H., and Phillips, P. H., *J. Nutrition*, 1941, **22**, 345.

[9] Shaw, J. H., and Phillips, P. H., unpublished data.

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